EFFECT OF QUERCETIN AND EPIGALLOCATECHIN-3-GALLATE ON ANTI-INFLAMMATORY ACTIVITY *IN VITRO* AND *IN* VIVO

A Thesis
by
KRISTA M KENNERLY

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APPROVED BY:

Dru A. Henson, Ph.D.
Chairperson, Thesis Committee

David C. Nieman, Ph.D.
Member, Thesis Committee

Andrew R. Shanely, Ph.D.
Member, Thesis Committee

Ted Zerucha, Ph.D.
Member, Thesis Committee

Zack E. Murrell, Ph.D.
Interim Chairperson, Department of Biology

Max C. Poole, Ph.D.
Dean, Cratis D. Williams School of Graduate Studies

Abstract

EFFECT OF QUERCETIN AND EPIGALLOCATECHIN-3-GALLATE ON ANTI-INFLAMMATORY ACTIVITY *IN VITRO* AND *IN* VIVO

Krista M Kennerly
B.S., Appalachian State University
M.S., Appalachian State University

Chairperson: Dru A. Henson, Ph.D.

The objectives of this study were two-fold: first, to elucidate quercetin's use as an anti-inflammatory neutraceutical *in vitro* by comparing supplementation of quercetin aglycone to physiologically relevant quercetin conjugates in lipopolysaccharide (LPS) stimulated human peripheral blood mononuclear cells (PBMCs) in culture. In addition, this study attempted to determine the optimal dose and combination of quercetin (Q) and epigallocatechin gallate enriched green tea extract (EGCG) required to optimize performance during consecutive days of exercise in mice.

For *in* vitro experiments, PBMCs ($1x10^6$ cells/ml), isolated from venous peripheral blood in healthy adults, were incubated (37°C, 5% CO_2) for 1 hour with various concentrations of either quercetin, quercetin-3'-sulfate (Q3'S), or quercetin-3-glucuronide (Q3GlcA) (1-50μM) dissolved in <0.1% DMSO, prior to 23 hours of LPS stimulation. A succinate dehydrogenase activity assay was then used to assess cell viability.

For *in* vivo experimentation, C57BL/6J mice (n=176) were randomly assigned to one of five groups: control, 6.25 mg Q/kg and 1.25 mg EGCG/kg (Q/EGCG low), 12.5 mg Q/kg

and 2.5 mg EGCG/kg (Q/EGCG mid), 25 mg Q/kg and 5 mg EGCG/kg (Q/EGCG high), and 25 mg Q/kg (Q). Mice were supplemented for 3 and 14 days (n=12 animals per group). For the last three days of the dosing cycle, mice were run on a treadmill at 14m/min for up to 2.5 hours. Cell culture supernatant and murine plasma concentrations of inflammatory cytokines were determined by either enzyme linked immunoabsorbant assay (ELISA) or using an electrochemiluminescence-based solid phase sandwich immunoassay.

Quercetin caused significant decreases ($P<0.05$) in LPS-stimulated TNF-α, IL-1β, and IL-6 secretion levels at concentrations equal to or greater than 25 μM, indicating an anti-inflammatory role of quercetin aglycone *in vitro*. However, physiologically relevant quercetin conjugates, Q3'S and Q3GlcA, failed to elicit the same significant effect. *In vivo*, plasma IL-10 was significantly reduced in all 14-day quercetin supplemented mice compared to control following exhaustive exercise. Despite this, quercetin supplementation failed to cause a reduction in any other plasma cytokine measure and failed to improve exercise performance in mice. Moreover, the addition of EGCG at a 5:1 ratio failed to augment any anti-inflammatory or performance enhancing effect.

In conclusion, while quercetin aglycone supplementation elicited a robust anti-inflammatory effect on human PBMCs *in vitro*, this effect was lost upon metabolic sulfation or glucuronidation. In mice, 14-day quercetin supplementation significantly decreased post-exercise plasma IL-10, but failed to alter any other cytokine measure or boost exercise performance, even upon addition of EGCG.

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Dedication

This master's thesis is dedicated to my mother, Helen P Wallenborn. Since my youth she has instilled in me the belief that I can accomplish anything despite the challenges that may face me. In this way, her strength and support have emboldened my endeavors in life.

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Chapter 1: Introduction

Nutritional immunology and its application in exercise science has been the endeavor of an increasing number of scientists over the past couple of decades. Interest grew following seminal studies that observed significant changes between resting and post-exercise immune measures[1]. This immunologic change culminates into transient immunosuppression following strenuous, exhaustive exercise and directly correlates with the physiological stress of an athlete. Epidemiological studies done on endurance athletes following elite competition suggest that this 'open window' of impaired immunity increases the risk for upper respiratory tract infections (URTIs)[2,3]. Despite the fact that the mechanism for this theory remains unsubstantiated, endurance athletes may benefit from neutraceuticals capable of mitigating potentially superfluous immune responses, such as inflammation, that develop during exhaustive competition.

To date the importance and specific function of the immune system in skeletal muscle recovery following exercise has been poorly understood. Important questions that remain unanswered are, what degree of inflammation incurred during exercise is necessary and when might it be detrimental to the resting health of an elite athlete. Many athletes self-prescribe nonsteroidal anti-inflammatory drugs (NSAIDs), like Ibuprofen, that can additionally exacerbate inflammation under certain conditions such as endurance events like marathons and ultra marathons[4,5]. Subsequently, research investigating alternative treatment options has intensified, especially with naturally occurring compounds, such as flavonoids, or their synthetic derivatives. The pharmacological potency of this phytochemical group was formally recognized in the early 1980s and since that time, numerous papers have been published on their effect on innate leukocyte function[6]. Quercetin is one such flavonoid that has been highly investigated in this manner, although claims on its effectiveness as an anti-inflammatory agent *in vivo* have been disparate and unsubstantiated in humans.

The purpose of the preliminary review herein was three-fold: first, to give the reader a thorough review of the current literature on the propagation of sterile inflammation in skeletal muscle, including specific roles for mast cells, neutrophils, and macrophages in this context. It should be

noted that it is beyond the scope of this thesis to discuss similar studies conducted on other innate cell types, such as dendritic cells and NK cells, and on leukocytes involved in humoral immune function. The next aim was to give a detailed description of the intercellular signaling pathway that activates canonical nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which is responsible for regulating expression of many of the pro-inflammatory genes in M1 macrophages. Lastly, this review will cover detailed descriptions of the flavonoids quercetin and epigallocatechin-3-gallate (EGCG), as well a comprehensive review of studies assessing quercetin's anti-inflammatory potential as assessed both *in vitro* and *in vivo*.

Chapter 2: Review of Literature

2.1 Systemic Immune Perturbations following Exercise

2.1.1 Immune status following intensive and prolonged exercise

It has been well established in the literature that heavy exertion during exercise has significant acute and chronic effects on the immune system. This can be especially appreciated in the differential level of circulating plasma cytokines measured pre- and post-exercise. Cytokines known to increase markedly after exhaustive exercise include interleukin (IL)-6, IL-10, IL-8, IL-1ra, granulocyte colony-stimulating factor, monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1β, tumor necrosis factor-α (TNF-α), and macrophage migration inhibitory factor[4,7–15]. Concomitant with these cytokine perturbations, an athlete undergoing intense and prolonged exercise, during which muscle microtrauma and oxidative stress are thought to be imminent, is said to develop suppressed immune function in respect to natural killer (NK) cell cytotoxicity, granulocyte respiratory burst activity, nasal and salivary immunoglobulin A (sIgA) secretion, delayed type hypersensitivity, and lymphocyte proliferative responses, as well as drastic changes in circulating immune cell populations[15–19]. The mechanistic cause of these immune perturbations in elite athletes, which can range between 3 and 72 hours, remains elusive, however some speculate that this "open window" in immune surveillance increases an athlete's susceptibility to bacterial and viral infection[20,21].

2.1.2 Open window theory

Several epidemiological studies support the "open window theory" of immunosuppression following exhaustive exercise, reporting increases in upper respiratory tract infection (URTI) risk in the one to two weeks following competitive endurance events[2,3,22]. Immediately following a Los Angeles marathon race, 12.9% of runners reported URTI incidences compared to 2.2% of control marathon runners not participating in the race, with incidence in participants increasing to 40% in the 2 months following the race. After controlling for various cofounders, it was determined that training more than 96 km/week increased URTI risk two fold compared to those training for less than 32

km/week[2]. In a subsequent study, increased URTI risk was noted in one out of four ultramarathon runners 2 weeks following a 160-km event[23]. Similar findings have also been reported in both human and mouse studies[22,24,25]. No studies suggested that any amount of endurance exercise could increase infection risk. On the contrary, out of the 2300 marathon runners training for the Los Angeles marathon, URTI risk wasn't significantly elevated until training distances exceeded 100-km per week[2].

Despite the overwhelming implication that significant immune perturbations observed post-exercise might put one at increased risk of URTIs, direct evidence linking these two phenomena is still lacking[26]. Of the notable immune dysfunctions experienced after rigorous exercise, the only potential markers that have significantly correlated with URTI risk have been relative low sIgA, a dominant immunoglobulin isotype on mucosal surfaces, and high IL-10 secretion from antigen-stimulated whole blood cultures[27–30]. Tomasi et al. (1982) were first to report this relationship in athletes after finding decreased sIgA in eight elite Nordic skiers competing in a national cross-country skiing event. Subsequent studies have reported this same phenomenon in athletes undergoing endurance events of at least 90 minute duration, including cyclists, marathon runners, and triathletes[31–34].

Despite sIgA output's ability to predict URTI risk on a group level, the level of individual predictive power is generally more suspect. Of runners completing a 160-km ultramarathon, sIgA secretion rate had an overall predictive value of 55%, since approximately half of non-URTI subjects experienced at least a 50% drop in sIgA output, proving to be false predictions. Other contributing factors need to be discovered before the predictive power of sIgA output can be established, since no other factors concerning demographic, training, or immune status have been able to account for this risk[23]. In short, substantiating the open window theory will require a better understanding of the immune system's response to exercise.

2.2 The Major Immune Players in Skeletal Muscle Damage and Repair

The immune system plays a pivotal role in exercise by mediating tissue repair and regeneration within skeletal muscle. For this to occur, concisely regulated crosstalk between immune cells and myofibers is played out in an adaptable orchestration of paracrine, autocrine, and contact interactions. Local acute immune responses at the site of injured myofibers can be divided into two phases, an inflammatory period, in which leukocytes repair damaged tissue by coordinating the removal of necrotic cells and their debris, and myofiber regeneration. Literature addressing the impact of necrotic fibers on specific immune cell subsets in response to exercise is limited, with many studies not exploring the intricacy of phenotypes that can exist within classically defined macrophages, dendritic cells, etc. However, models of acute injury do give a degree of insight into how innate immunity may function in the muscle microenvironment during an athlete's hypothetical open window.

Prior to discussing the literature on acute injury in murine skeletal muscle, a disclaimer must be said on relating these findings to exercise-induced inflammation in human studies. Many animal studies have provided evidence linking inflammation to exercise-induced muscle damage[35–39]. However, establishing this paradigm in humans has proven far more difficult and has recently been a topic of extensive review by Paulsen et al[40]. The biggest point of contention has been whether or not muscle damage is indeed occurring in various exercise protocols and if so what the most appropriate way to assess this damage is. The most direct approach has been histological analysis of muscle biopsies, however this can be unpleasant for study participants and at times unreliable since the procedure itself could incite inflammation. Moreover, assessing cytokine signaling and cellular interactions typical of inflammation using homogenized tissue could be problematic, since data would be presented as tissue- instead of cell-specific expression profiles. Animal models suggest that during muscular repair/regenerative processes the anatomical location and functional phenotype of immune cells can vary significantly at any given time, and to date no experimental procedures involving humans has been able to extensively explore this[40]. Currently, animal studies describe methods for obtaining viable cell populations by gentling digesting with 0.1% collagenase B followed by a

blocking step with anti-CD16 and anti-CD32 to preclude macrophage activation and adhesion to plastic[41].

In addition, it must also be pointed out that while human and murine immune systems are highly similar there are fundamental differences to be considered[42]. For instance, in humans plasma/serum creatine kinase (CK) activity peaks after 4-7 days[43–47], while animal models report this occurrence 1-3 days following contraction-induced, skeletal muscle injury[48–54]. It is also worth noting that while the predominant leukocyte in human blood are neutrophils (50-70% neutrophils, 30-50% lymphocytes), the prevalent leukocyte in mouse blood are lymphocytes (75-90% lymphocytes, 10-25% neutrophils)[55].

Furthermore, as the mechanisms of acute inflammation during muscle injury are discussed, bare in mind that immune responses that occur in response to exercise are variable and may not always reflect findings in acute injury models conducted in mice. In fact the majority of murine models used to investigate skeletal muscle inflammation on a cellular level represent the extreme in expected damage during exercise and include acute muscle damage from reloading after hindlimb suspension, injection of histotoxic compounds, ischemia-reperfusion [56–63]. At the other end of the spectrum models, such as relatively mild exercise-induced muscle damage (EIMD) protocols produce less of an inflammatory response with little to no neutrophil accumulation[51]. No studies to date have looked at the effect of heavy exertion during exercise on specific murine innate immune cell subpopulations *in vivo*. During exercise the extent to which muscle damage could occur is said to be relative to the intensity and duration of the activity, as well as individual training adaptations[4,15] Furthermore, extrapolating findings from acute muscle injury models is problematic due to the influence of exercise specific changes in stress hormones, stimulation of the sympathetic nervous system, body temperature changes, increases in blood flow, dehydration, diet, and supplement use on functional immunity[4,13,20,64,65].

2.2.1 Mast cells: Instigation of innate immunity in skeletal muscle

Necrotic cells resulting from acute injury within skeletal muscle initiate an inflammatory response involving marked immune cell infiltration into the affected tissue. Again, it should be stressed that the dynamics and magnitude of this response is dependent on the intensity and duration of the exercise performed[20]. In many ways this form of 'sterile inflammation' is similar to the classic inflammatory response instigated by microbes, however it does have important distinctions[66]. Principally, local activation of innate effectors is not incited by foreign pathogens, but instead by endogenous proteins and nucleic acids released from the apoptotic cells, which act as damage associated molecular patterns (DAMPs). DAMPs have been described in many necrotic tissue types and include heat shock proteins[67], S100 calcium-binding proteins[68], ATP[69], high mobility group box 1 (Hmgb1)[70], as well as mitochondrial formyl peptides and DNA[71]. Interestingly, several caspases integral in programmed cell death or apoptosis are capable of deactivating DAMPs. If this were not the case then the death of any cell within the body could potentially trigger an inflammatory response. Moreover, release of activated DAMPs from solely necrotic tissue allows sentinel immune cells (macrophages, dendritic cells, and mast cells) to specifically distinguish between necrotic death due to sterile injury or infection and homeostatic cell turnover [72].

One of the first instigators of sterile inflammation in skeletal muscle characterized by an immediate exudate neutrophil infiltrate is the resident mast cell. These cells are derived from $CD13^{+}CD34^{+}CD117^{+}$ (otherwise known as KIT) hematopoietic progenitors in the bone marrow and like monocytes, can only terminate their differentiation in peripheral tissue. Stimulation of resident mast cells can induce degranulation, release of eicosanoids, and expression of various chemokines and cytokines that elicit either adaptive or innate effects depending on the tissue-specific microenvironment[73].

During sterile injury, it has been shown that mast cells can specifically respond to ST2/IL-1RAcP ligand IL-33 released from necrotic structural cells. In addition to exogenous functions, this relatively new addition to the IL-1 family is said to have transcriptional regulatory properties and

associate with chromatin in the nucleus[74]. However, human skeletal muscle does not express IL-33 under steady state and its expression in contracting muscle is unknown[75]. Other known DAMPs released from skeletal muscle include HMGB1, uric acid, and adenosine. Yet supernatant from necrotic mouse embryonal fibroblasts (MEFs) isolated from HMGB1$^{-/-}$ mice, $A_{2A}R^{-/-}$ and $A_3R^{-/-}$ (adenosine specific receptors) mice, or treated with the uric acid production inhibitor allopurinol failed to influence mast cell activation. Despite the implications of these findings, the *in vitro* design of this study utilized phenotypically immature mast cells that may not recapitulate the mature phenotype *in vivo*[76].

Stimulation by exogenous ATP could also provide a putative mechanism for mast cell activation in skeletal muscle. ATP can not only act as a DAMP released from necrotic fibers, but its endogenous release during contraction is said to potentiate contractile force by acting on purinergic receptors resulting in calcium influx[77]. Exogenous release of ATP in this manner is also said to have autocrine effects resulting in increased glucose uptake and IL-6 expression[78,79]. Interestingly, it was recently shown that the ATP-sensitive P2X7-receptors on mast cells mediate initiation and exacerbation of intestinal inflammation, which was also characterized by a neutrophil infiltrate[80]. Whether or not exogenous ATP could have these same effects on mast cells residing in skeletal muscle is not known, but it is an intriguing possibility.

Even though the exact mechanism of mast cell activation in skeletal tissue remains elusive, it has been shown to be critical for the migration of short-lived neutrophils to the site of injured tissue. Indeed, inhibiting mast cell degranulation with the stabilizing agent sodium cromolate inhibited neutrophil infiltration into the skeletal muscle of rats by as much as 80% when challenged with bupivacain[63]. Mast cells along with some granulocytes are also said to be one of the first sources of IL-4, which can convert resident macrophages into a wound healing subtype.

2.2.2 Demolition tactics of the heavy-handed neutrophil

Neutrophils are critical, yet destructive innate effectors whose principle role in wound healing is microbial sterilization. These short-lived cells act as innate kamikazes in the battle against

infection, striking in as little as 15-45 minutes upon entry into damaged peripheral tissue. That is unless they encounter a bacterium, at which time they tacitly decide their fate by either starving the bacterium of essential factors that they require, such as iron, or delivering a payload consisting of peroxidase positive and negative granules, activation of their nascent phagosome, and the final pouring out their cytosol. They will even go as far as extruding their own chromatin in DNA nets laced with proteases from azurophil granules to vanquish their microbial prey.

Mast cell mediated neutrophil infiltration from peripheral circulation into the effected tissue occurs within minutes to hours after insult and requires extravastion across local endothelial vasculature. This occurs in four sequential steps involving selectin facilitated rolling, tight binding between leukocyte integrins and endothelium adhesion molecules, diapedesis via PECAM or CD31 expressed on both the leukocyte and at the intercellular junctions of endothelial cells, and chemokine mediated migration[81]. This general description of extravastion is followed by all exudate leukocytes, although the particular selectins, integrins, and adhesion molecules vary according to cell type. Neutrophils specifically require P- and L- selectin, integrin CD11b/CD18 (Mac-1), and intercellular adhesion molecule-1 (I-CAM, CD54). Upregulation of P-selectin on endothelial cells can be stimulated by histamine, leukotriene C_4 (LTC_4), or oxidants and upregulation of CD11b/CD18 on neutrophils is activated by platelet activating factor (PAF) or LTB_4, all of which can be generated by mast cells[82,83]. L-selectin is constitutively expressed on circulatory neutrophils and in combination with P-selectin facilitates rolling along the endothelium, however it must be shed before tight binding between CD11b/CD18 and I-CAM can occur. The neutrophil chemoattractant IL-8, also released from mast cells, has been shown to stimulate shedding of L-selectin[84]. Another important and unique feature of mast cells is their ability to retain stores of endogenous TNF-α. Mast cell degranulation and consequent release of TNF-α can in turn upregulate I-CAM, the ligand for CD11b/CD18 on the surface of vascular epithelium[85].

High plasma catecholamine, growth hormone, and cortisol levels have been responsible for increasing granulocyte count by up to 250% in the 3 hours following a high-intensity, endurance event[20,86]. If tissue damage occurs in exercising muscle during this time, activated neutrophils are certain to be a source of unrequited damage. Indeed, induction of lengthening contractions in extensor digitorum longus (EDL) muscles of mice deficient in CD18, a prerequisite for neutrophil diapedesis, had significant reductions in carbonyl content, a marker of oxidative damage, and histological and functional signs of muscle injury compared to wild-type[87] Despite their destructive potential, these cells are critical for staving off infection. Without them, non-sterile wounds tend to heal poorly and can cause lethality as evidenced in patients with full-thickness burns or deficiencies in microbicidal machinery. As key regulators in the inflammatory response, they are involved in recruitment, activation, and programming of antigen presenting cells (APCs) and serve as a source of chemokines attracting monocytes and dendritic cells (DCs) to the site of injury. In addition, their influence on macrophage polarization from pro- to anti-inflammatory states may be of unequivocal importance in myofiber regeneration[88,89]. However, the necessity for neutrophils in exercising muscle has yet to be shown and contraction injured $CD18^{-/-}$ mice retain macrophage recruitment comparable to wildtype, as well as faster restoration time of fiber size and force[87].

2.2.3 Macrophage polarization & facilitation of tissue repair

Macrophages are among the most essential of all innate effectors. They are the 'garbage collectors' of the body, clearing exogenous cellular waste from the interstium. Impressively, they are responsible for the clearance of around $2x10^{11}$ erythrocytes per day, which would equate to 'recycling' 3 kg of iron and haemoglobin per year. As phagocytes, these cells carry out their day to day functions independent of other effectors of the immune system. That is until the resident macrophage comes across debris indicative of the trauma or stress associated with DAMPs. Macrophages detect these endogenous danger signals via their toll-like receptors (TLRs), intracellular pattern recognition receptors, and the interleukin-1 receptor (IL-1R), most of which signal through the adapter protein myeloid differentiation primary-response gene 88 (MyD88). With this cue they

change their repertoire of surface proteins as they gear up production of pro-inflammatory mediators[90].

Macrophages differentiate from peripheral blood monocytes upon their entry into tissue either in the steady state or at the on slot of inflammation. Monocytes arise from myeloid progenitors in the bone marrow that diverge into various cell lineages, including neutrophils, eosinophils, basophils, macrophages, dendritic cells (DCs), and mast cells. In order for monocytes to emerge from this progenitor pool, they must first differentiate into monoblasts followed by their pro-monocyte precursors, only achieving monocyte status upon joining the rush of peripheral circulation. Although originating from the same precursors, not all monocytes are created equal and can extravasate at different stages of maturity, which may play a role in determining their functional phenotype within tissue. Murine monocytes at either end of this age continuum are termed 'inflammatory' and 'resident,' with the former spending the least amount of time in the bloodstream. Furthermore, these fairly evenly distributed populations can be identified according to the expression of particular cell surface markers. $CCR2^{+}$ (CC-chemokine receptor 2), CX_3CR1^{low}, $Ly6^{+}$ (also known as GR1) is said to designate an inflammatory monocyte and $CCR2^{-}CX_3CR1^{high}Ly6^{-}$ a resident monocyte[91]. In humans, these designations are $CD14^{high}CD16^{-}$ and $CD14^{+}CD16^{+}$ and are referred to as 'classical' and 'non-classical,' respectively[92]. It is interesting to note that in humans, the distribution of these populations is far from even, with the 'classical' monocytes making up approximately 90%[92]. So-called 'inflammatory' monocytes can further differentiate into either resident macrophages or DCs, while 'resident' monocytes continue to navigate the vasculature until an incidence of inflammation or trauma[91].

In mouse skeletal muscle, residential macrophages have been identified as $CD11b^{+}F4/80^{+}CD11c^{-}Ly\text{-}6C^{-}CX3CR1^{-}$[41]. In a homeostatic state these cells occupy the epimysium and perimysium connective tissue that sheath entire muscles and their fasciles, respectively. Upon recognition of tissue injury, they selectively release cytokine induced neutrophil chemoattractant (CINC or human IL-8) and monocyte chemoattractant protein 1(MCP-1 or CCL2). IL-8 is a ligand

for either CXCR1 or CXCR2 on neutrophils, basophils, CD8+ T-cells, and endothelial cells. MCP-1 or CCL2 is a ligand for CCR2 on monocytes, T cells, basophils, immature dendritic cells, and natural killer (NK) cells. Upon stimulation by TNF-α, endothelial cells can also secrete these two chemokines, resulting in the appearance of monocyte infiltration at 8 hours post injury, in addition to continued neutrophil and mast cell recruitment. After 24 hours, macrophages are the predominating immune cell in the affected tissue and persist for days.

Tissue macrophages derived from circulating CCR2+ monocytes are said to exist in a spectrum of phenotypes. M1 and M2 describe the extreme polarization of these phenotypes and have pro- or anti-inflammatory roles, respectively. More specifically, the M1 phenotype is characterized by the expression of inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6. IL-1β released by these macrophages will in turn stimulate parenchymal cells to release CXCL1 and CXCL2, chemokines that recruit more neutrophils to the affected muscle tissue[93]. The M2 phenotype has a far more diverse characterization, including expression of arginase-1, CD163 and mannose receptor, and/or anti-inflammatory cytokines such as IL-10. Macrophages derived from 'resident' monocytes have many important functions in resident tissues that include, phagocytosis of material (antigen, bacteria, injured or apoptotic cells), antigen presentation, secretion of pathogen fighting enzymes and oxidative derivatives, and production of growth factors and cytokines that influence parenchymal tissue, and immune cell recruitment.

2.2.4 Recently discovered roles for leukocytes in muscle regeneration

The role of immune cell infiltration into skeletal muscle is not limited to the production and resolution of inflammation for the removal of cellular debris. Indeed, regulation of stem and progenitor cells that either repair or replace damaged skeletal muscle tissue is dependent on this infiltrate. Until recently, mechanisms delineating exactly how this might occur were relatively unknown. In 2013, researchers out of University of California, San Francisco published a study describing an essential role for IL-4 and IL-13 signaling in muscle regeneration. They were able to show that upon cardiotoxin (CTX) induced injury, IL-4/IL13$^{-/-}$ mice developed persistent

inflammatory infiltrates and cellular debris in tibialis anterior (TA) muscle. Furthermore, the efficacy of the regenerative response in the skeletal muscle of these mice was impaired as evidenced by their significant relative uptake of Evans blue dye, which accumulates in damaged muscle fibers. In addition, histological examination of CTX injured TA muscles from IL-4/IL13$^{-/-}$ mice revealed a failure to regenerate centrally nucleated fibers compared to wildtype (WT). Immunostaining for desmin, a marker of mature myofibers, also confirmed an absence of regenerated muscle fibers in. To identify the source of IL-4 production in regenerating muscle, they utilized 4get reporter mice, which express green fluorescent protein (GFP) from the 3' untranslated region (UTR) of the endogenous IL4 gene. Using flow cytometry, they were able to gate on these GFP+ cells from injured muscle and noted that ~85-90% of these cell expressed markers indicative of eosinophils (Siglec F^{+}CD11b^{+}). Surprisingly, these results suggest that eosinophil and not myeloid derived type 2 cytokine signaling is required for the timely and complete regeneration of skeletal tissue[94].

Researchers out of Harvard University in Boston have also established a role for regulatory T (Treg) cells in potentiating skeletal muscle repair. They were able to punctually delete Tregs from skeletal muscle following CTX injury by utilizing a mouse line in which the diphtheria toxin receptor (DTR) was expressed under the control of forkhead box p3 (Foxp3) regulatory elements. Thus, upon diphtheria toxin (DT) injection, B6.Foxp3-DTR+ mice experienced specific deletion of Foxp3^{+}CD4^{+} cells, markers that identify Tregs. When these B6.Foxp3-DTR+ mice received DT either intraperitoneally or intramuscularly at the time of CTX injection, there was a larger CD45^{+} cellular infiltrate into skeletal muscle compared to DTR- mice, and myeloid cells within this infiltrate failed to transition between a primarily pro-inflammatory, Ly6c^{hi} to an anti-inflammatory Ly6c^{lo} phenotype. In addition, histological examination showed that there was a disorganized pattern of centrally nucleated fibers indicative of regeneration and Gomori's Trichrome staining revealed a substantive accumulation collagen indicative of fibrosis[95].

2.3 NF-κB: Master Regulator of Inflammation in Skeletal Muscle

In a matter of hours following injury, skeletal muscle accumulates both fluids and leukocytes from peripheral circulation, including neutrophils, eosinophils, and monocytes. Monocytes extravasating into inflammed tissue are immediately programmed into M1 macrophages, secreting a host of cytokines in a paracrine and autocrine manner, including TNF-α, IL-1β, and IL-6. These pro-inflammatory cytokines are all regulated at least in part by the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in macrophages[96]. TLR signaling is one of the most well-known ways of activating the intercellular signaling pathway that leads to NF-κB activation. Indeed, it is common to find *in vitro* assays assessing anti-inflammatory potential utilizing TLR ligands, such as lipopolysaccharide (LPS), to initiate NF-κB signaling. Section 2.3 will cover NF-κB signaling and its occurrence in both macrophages and myocytes where relevant.

2.3.1 Canonical and non-canonical NF-κB signaling pathways

Inducible NF-κB has been detected in virtually every cell type and is highly conserved among species. Mammalian NF-κB exists as either hetero- or homo- dimers composed of the proteins, RelA/p65, RelB, c-Rel, NF-κB1/p50, and NF-κB2/p52, the last two of which are derived from the precursor subunits p100, and p105 [97]. These proteins have the same 300-amino acid sequence termed the Rel homology domain (RHD), which is responsible for DNA binding, subunit dimerization, nuclear translocation, and physical interaction with any one of seven ankyrin repeat-containing inhibitors called IκB proteins. NF-κB predominately acts to promote transcription of its target genes, with transcriptional repression only occurring with p50/p50 and p52/p52 complexes[98].

There are two functional pathways in which NF-κB can undergo rapid activation and nuclear translocation, termed canonical and non-canonical. The former can be activated by a myriad of exogenous and endogenous factors, including ROS, growth factors, pro-inflammatory cytokines, genotoxic stress, viruses, and bacterial polysaccharides. Even in their inactive state, hetero- and homodimers of NF-κB can actively shuttle between cytosol and nucleus due to an exposed nuclear localization signal (NLS) on p50, which is not effectively masked by IκB protein[99]. Upon cell stimulation by any one of the aforementioned factors, activation of inhibitor of kappa B kinase (IKK)

will occur, which is made up of three subunits, IKKα and IKKβ, which are necessary for kinase activity, and IKKγ (NEMO), which serves as a regulatory protein[97]. Specifically, IKKβ of this complex functions to unmask the nuclear localization signal (NLS) on p65 by phosphorylating two amino-terminal serine residues (ser 32 and serine 36) on IκB, resulting in its polyubiquitination by an SCF family ubiquitin ligase and subsequent degradation by the 26S proteasome. This will permit p65 and p50 heterodimers to translocate into the nucleus where they bind to promoter and enhancer sites containing κB consensus sequence, GGGRNNYYCC, where R is a purine, Y is a pyrimidine, and N is any base[100]. Blocking this pathway in mice causes non-specific responses to infection and functional impairment in B-cells, and thus ascribes vital roles both innate and adaptive immunity[101].

In contrast to the canonical pathway, which has been found ubiquitously in most mammalian cell types, the non-canonical pathway is an alternative means of NF-κB activation found only in B-cells and is responsive to members of the TNF-receptor (TNFR) superfamily important in B-cell maturation and lymphoid organogenesis, including B-cell activator (BAFF), lymphotoxin-β (LTβ), CD27, and CD40 ligand[102–106]. When these ligands interact with their respective receptors on the surface of B-cells, it induces activation of an intracellular downstream kinase called NF-κB inducing kinase (NIK). This kinase will preferentially phosphorylate IKKα, which in turn will phosphorylate serine residues 866 and 870 on p100, leading to its polyubiquitination at Lys-855 and partial degradation into p52 by $SCF^{\beta TrCP}$ E3 ligase complex[107]. In its resting state, p100 is sequestered in the cytoplasm by the RelB subunit of NF-κB. However once processed, the newly formed p52 and RelB dimer undergoes nuclear translocation to regulate distinct target genes from the canonical pathway. Transgenic mice lacking the non-canonical form of NF-κB have severe functional defects in their B-cells and impaired formation of peripheral lymphoid organs[108,109].

2.3.2 Redox sensitive activation of NF-κB

It has been known for well over a decade that intracellular signaling that leads to transient NF-κB activation can be regulated by redox status in a cell type specific manner[110–119]. In fact, one of the first known exogenous activators of NF-κB was H_2O_2 in the early 1990s[120]. It is now known that

effective stimulation by either lipopolysaccharide (LPS) or proinflammatory cytokines like TNF-α and IL-1β not only require ROS, but also lead to enhanced production of ROS by concurrent activation of Rac1-NADPH oxidase complexes[121–123]. To this effect, decreases in intracellular GSH to GSSG ratio have been shown to correlate with increased IκBα phosphorylation and subsequent NF-κB activation[124,125], while antioxidants such as glutathione peroxidase[126] and N-acetyl-cysteine (NAC)[127,128] are known to effectively inhibit this pathway.

Cytoplasmic kinases in the NF-κB signaling pathway that are said to be redox sensitive include, IKK, mitogen-activated protein kinase/ERK kinase-1 (MEKK-1), and phosphoinositide 3-kinase (PI3K)/Akt[117]. MEKK-1 can potentiate stimulatory effects of either TNF-α or IL-1β on the canonical NF-κB pathway by directly interacting with IKKβ to mediate its site-specific phosphorylation[129,130]. Akt is a serine/threonine kinase with an inherent role in cell survival. It can also be activated by cytokines TNF-α and IL-1β, as well as LPS, following its recruitment to the cellular membrane in response to 3'phodphotylsyrf phosphoinositides generated by PI3K[131]. In a similar fashion to MEKK-1, Akt activation can lead to transactivation of IKKβ, but only in cells with a higher ratio of IKKα to IKKβ[132]. Furthermore, Akt may not be relevant in exercise-induced NK-κB activation since overexpression of MEKK-1, but not Akt, in a line of mouse myoblasts (C2C12) was able to potentiate DNA-NF-κB binding activity[133].

Oxidant status can not only affect NF-κB's translocation into the nucleus by redox regulation of cytoplasmic kinases, but can also directly impact NF-κB transcriptional activity within the nucleus. Redox sensitive proteins implicated in this manner of NF-κB regulation include protein kinase C ζ (PKCζ), cAMP-dependent protein kinase (PKA), mitogen and stress activated kinase-1 (MSK-1), ribosomal S6 kinase-1 (RSK-1), and casein kinase 2 (CK-2)[117]. However, NF-κB binding activity in the nucleus requires reduced conditions compared to oxidant-induced activation of pertinent cytosolic kinases.

2.3.3 NF-κB activation during exercise

Goodyear et al. (1996) first described a link between exercise and activation of redox-sensitive signaling pathways important to antioxidant gene expression. Results from this study described increased activation of JNK, ERK1/2, and p38 after treadmill running (20m/min, 10% grade) for up to 60 minutes in rats[134]. A year following this study, Sen et al. (1997) linked H_2O_2 treatment to the potent induction of NF-κB signaling in L6 muscle cells, results of which corresponded with intracellular GSH:GSSG status[135]. Similar levels of NF-κB binding were confirmed in rats after an exhaustive bout of exercise in rats. This study reported relative levels of NF-κB binding in both deep vastus lateralis (DVL, type II_a) and superficial vastus lateralis (SVL, type II_b) since important changes in metabolic and antioxidant function have been described in this muscle group during endurance exercise. Their results indicated that significant binding of NF-κB occurred in respective muscle types, peaking at 2 hours post-exercise and attributed transient activation of this transcription factor to the generation of ROS [136]. This same group would later go on to show both the activation and time course of other important members of the NF-κB signaling cascade as well, reporting significantly higher levels of p50 protein content, phosphorylated IκBα, and IKK in rats run for one hour or until exhaustion (25 m/min, 5% grade) compared to control. Peak activation of IKKα/β occurred within 15 minutes of exercise and IκBα phosphorylation remained elevated up until the immediate hour following exercise, with levels of p65 reaching their peak 2-4 hours post-exercise. These effects were significantly abolished by pyorrlidine dithiocarbamate treatment, a potent inhibitor of the 26S proteasome, and mimicked in a group of sedentary controls receiving lipopolysaccharide (LPS) prior to sacrifice [137]. Also, inhibiting either extracellular-signal regulated protein kinase (ERK) or p38 kinase effectively blunted contraction mediated IKK phosphorylation, especially when combined at 76 ± 5%[138].

Redox status can have a number of effects on transcriptional activity, many of which are species or tissue specific. It is hard to say how many genes are influenced by homeostatic regulation of muscle oxidation-antioxidant balance. Roles for NF-κB transcriptional regulation to this affect are

still putative and include upregulation of antioxidant genes to counter oxidative stress, pro-inflammatory genes critical for muscle regeneration following exercise, and genes important in cellular metabolism, including glucose transport, glycogen repletion, and lipid oxidation[139].

2.3.4 NF-κB regulation of endogenous antioxidant expression

Several important antioxidant enzymes are under at least partial transcriptional regulation by NF-κB in skeletal muscle, two of the most essential include mitochondrial superoxide dismutase (MnSOD) and glutathione peroxidase (GPX)[110,139]. MnSOD serves as the first line of defense against mitochondria derived ROS since it is responsible for converting superoxide radicals, escaping from complex I and III of the electron transport chain, to hydrogen peroxide (H_2O_2) and oxygen (O_2). There are three isoforms of SOD, 15-35% of which is active in the mitochondria and 65-85% remaining in the cytosol[140,141]. Evidence that this enzyme is actively upregulated by NF-κB during endurance training has been demonstrated in rats 48 hours post-exercise in type IIa, type I, and mixed type II by 80%, 54%, and 42%, respectively[142]. Type IIa and I muscle fibers in mice also exhibit higher levels of mitochondrial mass relative to mixed type II or IIb fibers[143]. In a subsequent study, observed increases in the transcription of MnSOD mRNA were only found in type IIa and not in type IIb immediately following exercise[136]. Increased expression of MnSOD has also been reported in human skeletal muscle both following acute submaximal exercise and 4 weeks of endurance training[144,145].

Expression of GPX is regulated both by NF-κB and AP-1, and of the 5 isoforms that exist in humans GPX1 and 3 appear to be functional in skeletal muscle[110,141]. These enzymes utilize GSH as an electron donor for reduction of either H_2O_2 or organic hydroperoxide (ROOH) into water (H_2O) and alcohol (ROH), respectively[146–148]. Similarly to SOD, active GPX has been found to be more prevalent in fiber types with high oxidative capacity that are recruited during submaximal endurance exercise. In fact, it has been reported that GPX activity can increase in response to endurance training, which can range from 20-177%[149]. By transfecting C2C12 cells with a trans-dominant inhibitor of IκBα, Zhou et al. [150] showed that GPX gene expression was inhibited by approximately

80% in response to H_2O_2. If these results can be extrapolated to human myocytes, then active NF-κB regulation of this enzyme would be essential in the upregulation of this essential, endogenous antioxidant enzyme.

2.3.5 NF-κB induction of pro-inflammatory factors

Canonical activation of NF-kB can induce transcription of a host of pro-inflammatory genes, such as interleukin (IL)-1, IL-2, IL-6, and TNF-α, as well as chemokines such as IL-8, macrophage inflammatory protein (MIP)-1α, monocyte chemotactic protein-1 (MCP1), regulated upon activation normal T-cell expressed and secreted (RANTES, CCL5), and eotaxin. Additionally, it can upregulate adhesion molecules essential to the propagation of the inflammatory response, including the intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM)-1, and E-selection, along with inducible enzymes such as COX-2 and iNOS, growth factors, some acute phase proteins, and immune receptors[151]. Sustained exercise is also known to induce expression of IL-8, and anti-inflammatory IL-10 and IL-1ra in circulating blood leukocytes[4,152,153]. To a degree, upregulation of these various factors is essential to facilitate post-exercise regenerative responses in damaged tissue and hypertrophic adaptations[154].

2.4 Prospective Use of Flavonoids to Improve Athletic Performance

The physiological effects of flavonoids as secondary, plant metabolites in humans have been of peak interest in the scientific community due to their putative antioxidant, antiviral, immunomodulatory, antithrombotic, antimicrobial, and anticancer action[155–160]. Interestingly, it wasn't until the early 1980s that the pharmacological potency of this phytochemical group was recognized, despite the fact that preparations of flavonoids had been used by laymen and physicians to treat human diseases for centuries prior to that time[6]. Since then, there has been increasing interest in the beneficial effects of polyphenols on human health and performance by both researchers and food manufacturers.

2.4.1 Classification and chemistry of quercetin and epigallocatechin gallate (EGCG)

Flavonoids, a major class of polyphenols, are distinguished by a fifteen carbon phenylchromane core made up of two aromatic rings, designated A and B, bound together by an oxygenated heterocycle, ring C. Flavonoid subclasses are differentiated according to their respective heterocycle, with numerous members making up each subclass due to the varying degree in which A and B rings are hydroxylated or methoxylated[161,162]. Quercetin and epigallocatechin gallate (EGCG) belong to the flavonol and flavanol subclasses, respectively.

Quercetin is present in plants in several different glycosidic forms, the most common of which is quercetin-3-rutinoside, also called quercetin-3-rhamnoglucoside or rutin. In onions, quercetin is bound to one or two sugar moieties, D-glucose or L-rhamnose, at the C-3 or C-7 position (quercetin-4'glucoside and quercetin-3,4'glucoside)[163]. In contrast, flavanols, including EGCG, are the only subclass of flavonoids that do not form a glycoside in plants and are extremely stable even upon exposure to boiling temperatures for 7 hours as long as pH does not rise above 6.5[164].

2.4.2 Occurrence of quercetin and EGCG in the human diet

Flavonoids are abundant micronutrients in the human diet with over 6000 identified in plants[165]. Fruits and beverages such as teas and wine are the predominant source of most polyphenols, with flavonols making up the most ubiquitous subclass at concentrations of ≈15-30 mg/kg fresh weight, found most commonly in onions (1.2g/kg fresh wt.), kale, leeks, broccoli, and blueberries. In the United States, the estimated intake of flavonols and flavones among health professionals was 20 to 22 mg/day in 2002, of which 73% and 76% was quercetin in men and women, respectively[166]. Dietary sources of quercetin are commonly tea, red wine, fruits, and vegetables[167,168]. Functional foods made up of 0.008-0.5% or 10-125 mg/serving of quercetin have been used as nutraceuticals.

Attempting to get recommended nutraceutical doses of particular flavonoids from diet alone remains a challenge due to many factors affecting polyphenol content in foods, such as ripeness at the time of harvest, environmental factors, processing, storage, and culinary preparation[161]. Biosynthesis

of flavonols in plants is directly stimulated by light, which is why they are typically localized in the outer and aerial tissues. Accordingly, concentrations of quercetin can vary from plant to plant or even throughout a single piece of fruit based on exposure to sunlight[169]. Other environmental effects that can influence polyphenol content include other pedoclimatic factors, such as soil type and rainfall, and agronomic factors, such as being planted in a greenhouse versus a field, biological and hydroponic culture, and fruit yield per tree. The most significant loss of polyphenol content likely occurs during culinary preparation. Since most polyphenols are located in the skins or outer portions of fruits and vegetables, simply peeling these foods could eliminate them from a meal. Cooking can also have a significant impact on flavonoid content. After boiling tomatoes or onions for approximately 15 minutes there is a marked reduction in quercetin content anywhere from 75 to 80%, 65% after microwaving, and 30% after frying[170]. Fortunately, quercetin is also available as a dietary supplement in many countries including the US at a recommended dosage range of 200-1200 mg/day[171]. All toxicity data to date on quercetin's use in either respect support its safety in humans[171,172].

2.4.3 In vivo fate of quercetin and EGCG

Before discussing the therapeutic potential of either quercetin or EGCG in humans it is important to know their *in vivo* fate. Bioavailability of both the aglycone form of quercetin and the plant-based glycoside will be addressed to note any differences between obtaining this flavonoid from functional foods versus supplementation.

In general, polyphenols are metabolized via a common pathway[173]. Hydrophilic glycosides are hydrolyzed at the brush border of the intestines by intestinal enzymes or colonic microflora. Efficiency of absorption is significantly lower when bacteria are involved because subsequent aglycones are typically further degraded into simple aromatic acids. Aglycones that withstand degradation undergo conjugation involving methylation, sulfation, and glucuronidation both in the small intestine and the liver after transport via the hepatic portal vein. The metabolic processes associated with the conjugation of flavonoids are otherwise known to decrease the toxic and

biological effects of compounds like xenobiotics, as well as increasing the hydrophilicity required for their biliary and urinary excretion[161].

Initially it was thought that intestinal absorption of quercetin glycosides could only occur after hydrolytic enzymes from colonic microflora released the aglycone[174,175]. This was a reasonable assumption since intermembrane transport proteins specific for flavonoids had yet to be identified, and according to the rule-of-five prediction model, hydrophilic compounds with a molecular mass greater than 500 are incapable of passive diffusion across enterocytes[176]. Additionally, after administering 4 grams of quercetin to 6 volunteers Gugler et al. (1975) failed to detect it in either plasma or urine[177]. Quercetin glycosides are now known to be absorbed in the small intestine after hydrolysis by either brush border enzymes, such as lactase phloridzin hydrolase and cytosolic β-glucosidase, or colonic microflora[178–180]. If, however, quercetin is ingested in its aglycone form then it can also to some extent undergo absorption flux from the gastric wall of the stomach. When a 15 μmol/L concentration of quercetin was injected into the gastric lumen of rats, only 62% of that administered dose was recovered in the gastric content, with absorptive flux by the gastric wall reported at 1.11±0.08 nmol/min[181]. Flavanols, such as EGCG, are also present in plants solely in an aglycone form (without a sugar moiety), however depolymerization has not been reported within the stomach. In fact, when six healthy, human subjects were allowed to drink a flavanol rich cocoa beverage no change was observed in the HPLC profile of procyanidins, oligomeric compounds formed from catechins and epicatechin molecules[182].

Intestinal uptake and subsequent biliary excretion of quercetin glycosides varies according to their respective sugar moiety, but not the position at which it is attached to the quercetin molecule. Following a 30-minute *in situ* intestinal perfusion in rats, quercetin-3-O-glucoside and quercetin-4'O-β-glucoside were readily absorbed and corresponding peripheral plasma and portal vein metabolites recovered. However, when the same experiment was run with quercetin-3-O-β-galactoside, quercetin-3-O-rhamnoside, and quercetin-3-O-α-arabinopyranoside they were resistant to hydrolysis and only trace amounts of metabolites were detected[183]. Quercetin glucosides are also absorbed more

readily then the aglycone form of quercetin when taken orally. When nine healthy ileostomy subjects were given diets of either fried onion made up predominantly of glucosides, quercetin rutinoside, or quercetin aglycone, absorption defined as oral intake minus ileostomy excretion, was 52±14%, 17±15%, and 24±9%, respectively[184].

The most common way EGCG is administered in human clinical trials is within green tea or green tea extract, which also contains other active catechins, including epigallocatechin (EGC) and epicatechin (EC). Despite similarity in chemical structure, these catechins display differing pharmacokinetics, with EGCG displaying the poorest bioavailability. When green tea was administered in three repeated experiments to eight human subjects, plasma concentrations of EGCG, EGC, and EC were 77.9±22.2, 223.4±35.2, and 124.03±7.86 ng/ml, respectively[185]. Interestingly, Zhu *et al* (1997) looked at the effect of pH on the stability of these catechins since there is a rapid change in pH from the stomach to the intestine and found that EGCG was preferentially destroyed in solutions with pHs over 6.5, while EC and ECG were relatively stable[164]. The low bioavailability of EGCG is significant in terms of its potential therapeutic use since most published cell culture studies use concentrations of EGCG in the 10-100 μM range at pH 7.4[186].

The relative importance of conjugation, be it methylation, sulfation, or glucuronidation, appears to be dependent on the type of substrate (aglycone or type of glycoside) and the dose ingested. Typically, when quercetin is ingested as a nutraceutical in functional foods, conjugation of the aglycone after hydrolysis in the small intestine will occur immediately by small intestinal microsomes in the -3-, -7-, -4'- and -3'-position[187]. However, if the concentration of aglycone is high enough, as can be the case with pharmaceutical doses, conjugating enzymes in the small intestine become saturated and free (unconjugated) quercetin will pass into the hepatic portal vein in route to the liver. The principle conjugates leaving the small intestine are quercetin-7-glucuronide and quercetin-3-gluronide. If incubated with HepG2 cells, used as an *in vitro* hepatic model system, quercetin-7-glucuronide was metabolized by either methylation in the 4'- and 3'-position (catechol moiety) or deglucuronidation by β-glucuronidase followed by sulfation. A similar metabolism profile

can also be said for quercetin-3-glucuronide with methylation and sulfation accounting for 32% and 10.2% of quercetin in media after 48 hours, respectively. In contrast, quercetin aglycone yielded four classes of metabolites that were qualitatively similar to those from quercetin-3- and quercetin-7-glucuronides, but the rate of metabolism occurred faster over a 24-hour period[188]. The same metabolites for this study have also been reported in rats, rat hepatocytes, and human plasma[189–191]. Interestingly, EGCG is one of the few flavonoids that predominantly escapes conjugation in the liver and small intestine and instead can be found in free (unconjugated) form in human plasma hours after ingestion[185,192].

Following absorption in the small intestine and metabolism in the liver, bioavailability of flavonoid metabolites in peripheral tissues is dependent on both their binding within plasma and their elimination route from the body. From the liver, quercetin conjugates have an extremely high affinity (>99%) for the plasma protein albumin in systemic circulation, which may result in poor tissue deposition[193]. Alternatively, it may however delay the clearance of quercetin metabolites from the body, providing a way station, so to speak, for their distribution to target organs[194]. Significantly, elimination of quercetin metabolites in humans is quite slow, with half-lives ranging from 11 to 28 hours[195]. Methylated and/or sulftated and/or glucuronidated metabolites are polar, water-soluble compounds and thus can be excreted via either bile or urine. How the particular metabolite is conjugated does influence the elimination pathway, with glucuronides secreted in bile to a higher degree then sulfates, which are typically eliminated via urine[196]. If metabolites are secreted in bile then they end up in the duodenum, where they are further hydrolyzed by bacteria in the large intestine. The resultant products may be reabsorbed back into the body and undergo enterohepatic cycling[197]. Compared to the relatively slow elimination of quercetin metabolites, catechins such as EGCG have relatively fast-half lives[195].

The metabolic fates of quercetin could also differ between humans and mice. Indeed, a study measuring quercetin in both human and mouse plasma found that a large proportion of quercetin was methylated to isorhamnetin (up to 48%), while only low amounts of isorhamnetin could be detected

in the human plasma samples. The general conclusion from this data was that differences in the hepatic conversion of quercetin into its methylated metabolite isorhamnetin might differ between mouse and human. However, it is also possible that the quercetin dose was responsible for this effect, since the dietary quercetin concentrations used in the study (0.05-2mg/g diet) corresponded to 10-400 mg quercetin per kg body weight, which is several times higher than quercetin concentrations that can be achieved in humans[198]. An even more dramatic difference in flavonoid metabolism between species has been described for EGCG. In mice, it has been reported that 50-90% of EGCG present in plasma was conjugated, which differs from results reports in humans in which plasma EGCG was largely unconjugated[185,192,199].

2.5 Quercetin's Effect on Inflammatory Response: *in vitro* studies

Quercetin is among one of the most highly investigated flavonoids for its potential use as a performance enhancing nutraceutical. The most obvious reason to link quercetin supplementation with improved athletic performance is its ability to scavenge oxidizing species created during intense and prolonged exercise. In fact, many flavonoids, particularly those containing a catechol structure on their B-ring (3'4'dihydroxyl), have proven to be efficient antioxidants[200,201]. As will be evident later in this review, there are numerous *in vitro* and *in vivo* studies that provide convincing evidence of putative therapeutic benefit. Epidemiological data also lends support to the idea that a quercetin rich diet aids in disease prevention, including reduced risk of colorectal, kidney, pancreatic, prostate, lung, and breast cancer, cardiovascular disease, and diabetes mellitus[202–211].

Early studies looking at the effect of quercetin on macrophage function date back to the late 1990s. At this time, the plasticity and functional polarization of tissue macrophages was just beginning to be appreciated, with the concept of M1, M2a, M2b, and M2c polarization not being formally established until 2004[212]. Early *in vitro* study design typically utilized the commercial murine RAW 264.7 or J774A.1 monocyte cell line, which could be further differentiated into what was termed classical macrophages upon stimulation with LPS or cytokines such as INF-γ or TNF. Today we would recognize this type of activation as geared towards an M1 macrophage fate[213]. Two

studies published in 1999 utilized this method to make the seminal finding that incubation with quercetin at concentrations ranging from 50-200 μM was able to significantly inhibit TNF-α and nitric oxide (NO) secretion from these cells[214,215].

The finding that quercetin can inhibit nitric oxide production in murine macrophages has been described in numerous studies[214,216–227]. However, quercetin's capacity to inhibit TLR mediated inducible nitric oxide production is in itself not relevant in humans. Both mice and human macrophages are capable of TLR2 activation and subsequent antimicrobial response, but iNOS inhibitors L-NIL and L-NAME can only ablate this activity in mice, not humans.[228] Instead, TLR signaling in human macrophages up-regulates expression of both the vitamin D receptor and vitamin D-1-hydroxylase genes, which in turn leads to the induction of the antimicrobial peptide cathelicidin[229].

Many studies reporting quercetin's potent anti-inflammatory potential in macrophages cite its effect on prostaglandin E_2 (PGE_2) and a quintessential enzyme involved in PGE_2's production, cyclooxygenase-2 (COX-2)[217–221,225,230–232]. COX-2 is an enzyme capable of converting arachidonic acid sequestered in the plasma membrane to prostaglandin H_2. (PGH_2), which is in turn converted to one of four types of prostanoids, including PGE_2, by various terminal synthases[233]. Starting in 2001, studies have reported quercetin's effect on COX-2 expression in macrophages stimulated with LPS *in vitro*, but this effect was only significant at or above concentrations of 50μM[217,218,225]. Achieving such a concentration of quercetin in human plasma is likely unrealistic, with the highest reported average to date at 20.97μM[234]. Quercetin's ability to inhibit PGE_2 production is however far more impressive, with as little as 0.5μM required to significantly decrease expression LPS stimulated J774A.1 macrophages[217]. The disparity in quercetin's effect on PGE_2 compared to COX-2 expression is important to note in the context of skeletal muscle. During exercise, induction of prostaglandin expression is normal and positively correlates with exercise intensity[235]. COX-$2^{-/-}$ mice are reported to have an impaired response to stretch, which normally stimulates myoblast production of PGE_2,

$PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$ contributing to vasodilation and hyperaemia in skeletal muscle[236–239]. Following injury these effects are exacerbated in NSAID treated or COX-$2^{-/-}$ mice to the extent of delayed muscle regeneration, due to a reduced capacity for myoblast proliferation, differentiation, and fusion[240–242]. On the other hand, excessive levels of PGE_2 in skeletal muscle have been linked to inflammatory myopathies[243–247]. For instance, levels of dietary, saturated fatty acids, such as that found in patients with type 2 diabetes mellitus, have been linked to inflammation potentiated by excessive levels of PGE_2 in skeletal muscle[248]. The point at which PGE_2 stops being solely beneficial to muscle recovery and begins to exacerbate an ongoing inflammatory response in muscle is unknown, but it is certainly possible that it could occur during intense and prolonged exercise. In that case, a supplementing agent that can dampen PGE_2 expression without effecting basal prostanoid production could have a positive impact on muscle performance and recovery.

To date, the majority of macrophage studies reporting quercetin's anti-inflammatory potential have looked at its influence on NF-κB activation and downstream regulation of pro-inflammatory gene expression. As discussed previously, the NF-κB heterodimer RelA (p65) and p50 subunits are activated in the canonical signaling pathway following inflammatory stimulus from bacterial antigen and/or pro-inflammatory cytokines, such as LPS and TNF-α. Once this signaling pathway is active in macrophages, the p65/p50 NF-κB heterodimer translocates to the nucleus where it regulates expression of a host of different genes, including many that are essential to the development and/or persistence of an inflammatory response. Cytokine data from LPS stimulated macrophage cultures was the first and most reproduced evidence that quercetin could have an inhibitory effect on canonical NF-κB signaling. Under these conditions, pretreatment with 3 up to 100 μM quercetin has been reported to significantly decrease transcription of TNF-α, while anywhere from 1 to 50 μM was been reported to significantly decrease TNF-α secretion[214,220,222,223,227,249–253,253,254]. Similar findings have also been made for IL-1β, with 3 to 100 μM quercetin sufficient to inhibit transcription and 5 to 10 μM quercetin significantly inhibiting protein secretion[220,222,227,249,254]. Other factors under NF-κB

regulation that quercetin is reported to inhibit include CXCL10, IL-6, IL-8, and CCL3[220,227,249]. The variance in quercetin concentration required to block cytokine expression in these studies is important to note, and can likely be explained by differences in experimental design.

In order to define the mechanism through which quercetin inhibits TNF-α and Il-1β production in macrophages, several studies have investigated whether or not it has a direct effect on canonical NF-κB signaling. The most convincing and reproducible evidence to support quercetin's specific action on this pathway lies in its ability to inhibit LPS induced phosphorylation and subsequent degradation of the NF-κB inhibitory protein IkBα[222,223,249,250]. IkBα partially masks the nuclear localization sequence of NF-κb and it itself contains a nuclear export sequence that can rapidly translocate the transcription factor out of the nucleus[97]. Few studies have looked upstream of the transcription factor itself to determine where exactly quercetin may be inhibiting canonical signaling. However, a comprehensive study published in 2013 was able to show that 20 μM quercetin was sufficient to inhibit degradation of TRAF-6 and reduce downstream phosphorylation of TAK-1 and IKKα/β. In addition, Endale *et al*. proposed that quercetin exerts its inhibitory effects on NF-κB signaling through modulation of PI3 kinase activation resulting from the inhibition of tyrosine kinase Syc[255]. However, the relevance of Syc and PI3 kinase in LPS activated TLR4 signaling remains a matter of debate[256–260].

2.6 Quercetin's Effect on the inflammatory response: *in vivo studies*

Animals models have proven to be a useful tool in understanding the effect of quercetin treatment *in vivo*. Oral administration of quercetin was shown to increase exercise tolerance[261–263], voluntary activity[263], markers of mitochondrial biogenesis[262,263], and prevention of cardiac muscle damage[261]. Furthermore, quercetin reduced risk of upper respiratory airway infection in mice after stressful exercise upon exposure to influenza[264].

2.6.1 Seminal studies using quercetin supplementation in athletes

Several *in vivo* studies have been performed in order to clarify the role of quercetin in humans under conditions of exercise induced physiological stress. In a randomized double-blind placebo-controlled clinical trial of 26 male untrained badminton players, time to exhaustion was significantly decreased in athletes supplemented with 1000mg per day of quercetin for eight weeks[265]. In another cohort of untrained males, supplementation of quercetin for two weeks (1000mg) significantly improved treadmill time trial performance[266]. However, two independent studies failed to measure any benefit of quercetin supplementation (1000mg) on the performance in trained cyclists[267,268]. The disparities observed between these studies could be due to quercetin having greater influence on mitochondrial biogenesis in untrained compared to trained athletes.

In a randomized placebo controlled study of exhaustive exercise, athletes cycled at 57% $Watts_{MAX}$ for 3hrs daily on three consecutive days. Cyclists supplemented with 1000mg of quercetin for five weeks had significantly increased plasma quercetin levels compared to the placebo[8,12,269]. Despite increased plasma quercetin levels, supplementation failed to attenuate increases in plasma cytokines post exercise. Furthermore, muscle NF-κb did not increase post exercise and no difference was observed between the quercetin supplemented and placebo groups[8,269]. Despite no observable difference in immune response following exercise, quercetin supplementation did markedly reduce upper respiratory tract infections in the two week period following the three day exhaustive exercise program (placebo 45% Vs. quercetin 5%)[12]. Similar results were obtained by the same research group, in an additional study investigating quercetin supplementation in endurance runners. Runners in the western states endurance run were randomized with either placebo or quercetin (1000mg) for three weeks prior to the 160km event and for the two weeks following the race. There was no significant difference in inflammatory cytokines, performance or immune response in athletes supplemented with quercetin compared to the placebo control group[270–272]. While a trend in the reduction URTI's was observed in the quercetin supplemented group, this reduction failed to reach

significance[273]. The authors attribute the lack of significance to the relatively small study size and an overall reduced incidence of UTRI as a result of season variation.

2.6.2 Modulation of quercetin supplement formulation

Data from those founding studies conducted on quercetin supplementation *in vivo* failed to live up to the standards set by both the flavonoid's potent anti-inflammatory action *in vitro* and optimistic findings in animal studies. Of chief concern was the poor bioavailability of quercetin aglycone in humans, with levels of plasma quercetin obtained from study participants often falling below the 5μM minimal concentration that has been shown to significantly reduce TNF-α levels in PMA stimulated human PBMC culture[8,250,254,266,269,273–275]. To combat this issue, quercetin supplementation was reformulated with other flavonoids and food components that could potentially improve quercetin's bioavailability and bioactive function[225,226,276–281]. Additions to the supplement included another flavonoid epigallocatechin 3-gallate (EGCG)[280,282,283] from tea, a glycosylated form of quercetin called isoquercetin (quercetin-3-glucose or hirsutrin)[195,276,284], N_3polyunsaturated fatty acids (N_3-PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)[276], and the nutrients vitamin C, vitamin B3, and folate[279,281].

To test this newly formulated supplement, a total of 39 trained cyclists were recruited and asked to take Q-EGCG, Q-chew (Q-EGCG minus EGCG, isoquercetin, and N_3PUFA), or placebo soft chew supplements twice a day for a total of 14 days. Following supplementation, participants cycled for 3 consecutive days (3 hours per day) at ~57% W_{max}. Cyclists on both Q-EGCG and Q-chew had significantly higher levels of plasma quercetin pre-, post-, and 14 hours after exercise compared to placebo, with Q-EGCG trending at higher levels then Q-chew[285]. Heightened plasma quercetin levels in Q-EGCG users correlated with changes in peripheral immune status during recovery, as evidenced by reduced total blood leukocyte counts, a 50% reduction in serum CRP, and a decrease in plasma IL-6 levels that were 39% less then placebo. In addition, both Q-chew and Q-EGCG groups had a significant decrease in plasma levels of IL-10 and G-CSF, post- and 14 hours post-exercise, respectively. Importantly, the observed changes in immune status observed in quercetin

supplemented athletes coincided with reduced delayed onset muscle soreness (DOMS) scores at day 2 and 3 of recovery. Despite these encouraging findings, it should be noted that not all immune perturbations measured in this study were countered by quercetin intervention, since expected rises in plasma IL-1ra and MCP levels failed to differ between treatment groups[268,286]. Due to the experimental design employed in the Q-EGCG study it was not possible to delineate which ingredient or combination was responsible for the observed changes in immune status between treatment groups[285].

2.6.3 Controversy over nutraceutical anti-inflammatory use in athletes

Even if direct evidence substantiating the "open window theory" were discovered there are those that criticize the use of nutritional supplements to dampen the degree of physiological stressors, such as inflammation and oxidative stress, that are thought to accompany infection risk. The principle argument against such interventions suggest that it would interfere with important signaling pathways and gene expression essential to adaptations made during exercise training[144,145,287]. This concern is understandable since many of the dietary supplements in question have potent antioxidant potential and could inhibit redox-sensitive pathways such as MAPK, which is said to regulate growth, metabolism, differentiation, and remodeling of skeletal muscle[288]. Similarly, inhibition of NF-κB is also questioned due to its regulation of gene products involved in inflammation and protein turnover[289]. Also, as previously mentioned, both of these transcription factors upregulate endogenous antioxidant enzymes, such as MnSOD, copper-zinc superoxide dismutase (CuZnSOD), GPX, and iNOS[110].

Another issue is that either one of these pathways may regulate expression of the so called "myokine" IL-6[290]. *De novo* synthesis of IL-6 in skeletal muscle has been reported in variety of conditions, including exercise[291,292], muscle inflammation[293] and microtrauma[294], hypoperfusion[295], and following denervation[296]. Its status as a myokine prototype is said to be especially notable during exercise since it precludes other circulating cytokines in both onset and relative abundance[290]. Pedersen et al. [290] has theorized that regulation of IL-6 expression in myocytes is unlike that in

macrophages, which is predominately regulated by NF-κB. Instead it was suggested that IL-6 signaling in muscle was seemingly more complex implicating both Ca^{2+}/nuclear factor of activated T-cells (NFAT) and glycogen/p38 mitogen-activated protein kinase (MAPK) pathways in its regulation. However, a more recent study has more definitively shown that electric-pulse-stimulation (EPS) of C2C12 myotubes specifically activates c-jun terminal kinase (JNK) and activator protein 1 (AP-1), with inhibition of JNK completely abolishing all EPS-induced increases in IL-6 gene and protein expression. Therefore, inhibition of classical inflammatory pathways in leukocytes or myocytes alike during exercise may have no effect on the regulation of IL-6. This is an important point to make since IL-6 is thought by many to play a beneficial role during exercise by increasing glucose production in the liver and lipolysis in fat tissue[297]. In addition, IL-6 present in circulation during exercise has been identified as the trigger for IL-10 and IL-1ra in blood leukocytes[153]. IL-10 inhibits production of a multitude of inflammatory cytokines[298–300] and IL-1ra functions to inhibit IL-10 receptor binding[301].

Interestingly, a recent study by Marklund et al.[302], conducted on nine experienced athletes participating in a 24-hour ultra-endurance event provided evidence to suggest that intensive exercise increases expression of major histocompatibility complex I (MHC I) in both the cytoplasm and sarcolemma of skeletal muscle fibers[303]. Upregulated MHC class I expression is traditionally a pre-requisite for antigen-specific CD8 mediated cytotoxicity, and thus is not expressed at detectable levels in normal human skeletal muscle[303–305]. In addition, they also found a significant inflammatory cell infiltration in the skeletal muscle, with a 2-3 fold higher number of $CD3^+$, $CD8^+$, and $CD68^+$ cells[302]. Prior to this study only three other studies have addressed local skeletal muscle inflammation in response to endurance exercise, only two of which reported inflammatory infiltration into skeletal muscle following marathon running[306–308]. Additionally, increased expression of MHC class-I in the sarcolemma of muscle fibers has previously only been shown in autoimmune muscle diseases that result in muscle cell damage and myositis[305,309–312]. This leads one to speculate as to whether increased MHC class-I expression with an accompanied infiltration of inflammatory cells could be

responsible for the typical muscle damage procured during sustained, intense exertion of marathon and ultramarathon races. In support of this idea, a study conducted in 2000 found biochemical and histological features of myositis after over-expression of a mouse MHC I gene, H-2K^{b}, by linking it to a constitutively active creatine kinase promoter[313]. If substantial leukocyte infiltration obtained during sustained, endurance exercise is the causative agent of increased infection risk in these elite athletes, then it would seem appropriate to target traditional inflammatory pathways, such as NF-κB, in an attempt to blunt the detrimental effects of this superfluous inflammation on an athlete's health and performance.

2.7 Purpose

As previously discussed, there are a number of *in vitro* studies that have linked quercetin's anti-inflammatory capabilities to the modulation of signaling pathways vital to cell function. These actions include a diminished release of pro-inflammatory cytokines by inhibiting cell signaling that would otherwise lead to the activation of the transcription factor NF-κB. However, no study has analyzed the anti-inflammatory activity of the *in vivo* metabolites of quercetin *in vitro* using human peripheral blood mononuclear cells (PBMCs). The *in vitro* study utilizing human PBMCs detailed in this thesis will be the first to characterize the anti-inflammatory properties of two quercetin metabolites, quercetin-3'-sulfate and quercetin-3-glucuronide, incorporating levels mimicking those found in human blood plasma after digestion of quercetin. Findings from this *in vitro* study may help explain why quercetin failed to counter exercise-induced inflammation in preliminary human studies.

Using the same *in vitro* model, quercetin and 45% EGCG enriched green tea extract will be tested both alone and in various combinations to determine the optimal amounts and proportions. The goal will be to develop a quercetin-green tea extract supplement that exerted a strong *in vitro* anti-inflammatory effect, which could then be tested *in vivo*.

Building on results found in the *in vitro* study, an additional animal study will be conducted to determine the optimal dosing regimen with a quercetin-green tea extract supplement to counter exercise-induced inflammation. Experiments with mice will be designed to test the amount and

duration of quercetin-green tea extract supplementation that exerts the best anti-inflammatory influences post-exercise.

Chapter 3: Materials and methods

3.1 *In Vitro* Study

3.1.1 Peripheral Blood Mononuclear Cell (PBMC) isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-treated venous peripheral blood harvested from healthy, male and female human volunteers (age 18-30) by a low-density iodixanol density barrier method. The institutional review board (IRB) of Appalachian State University approved the protocol for the collection of human blood in this study (#10-0185). All participants were fully cognizant of the aim and details of the study and gave their written informed consent.

PBMC isolation was performed using a low-density iodixanol density barrier method. This was accomplished by first adjusting the density of plasma in whole blood to $\rho = 1.095$ g/ml with a working solution (WS) of 40% (w/v) iodixanol, a 1:3 dilution of Optiprep® Density Gradient Medium (Sigma Aldrich, St. Louis, MO, USA) in 0.85% (w/v) NaCl and 30 mM Tricine-NaOH, pH 7.4. Aliquots (5 ml) of this suspension were layered underneath equivalent volumes of $\rho = 1.078$ g/ml density barrier solution, a 1:2.92 dilution of WS in tricine buffered saline (0.85% NaCL, 10 mM Tricine-NaOH, pH 7.4). Density separation was achieved by centrifugation at 700xg and 20°C for 30 minutes with slow acceleration and no brake. Resolved, mononuclear cell fractions (1.068 g/ml layer) were collected and washed twice at 400xg and 20°C for 10 minutes, with normal acceleration and deceleration, in RPMI-1640 medium (Sigma-Aldrich, Saint Louis, MO, USA) containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA) supplemented with 200 mM GlutaMAX (Life Technologies, Grand Island, NY, USA); 10,000 U/ml & 10 mg/ml penicillin streptomycin, respectively (Sigma Aldrich, Saint Louis, MO, USA); and 100mM sodium pyruvate (Sigma-Aldrich, Saint Louis, MO, USA) (C-RPMI).

3.1.2 Flavonoid preparation

Quercetin, purchased from Sigma-Aldrich (Saint Louis, MO, USA), was dissolved in dimethyl sulfoxide (DMSO) before being diluted in C-RMPI. DMSO is a known antioxidant capable of scavenging hydroxyl radicals[314]. Therefore, to eliminate any confounding effects DMSO may have on PBMC inflammatory status, quercetin stocks were prepared at 1324-fold of each respective concentration used in culture, such that DMSO was <0.01% and consistent across treatments. An equivalent concentration of DMSO was also included in all controls and treatment groups lacking quercetin. Dulbecco's phosphate buffered saline (PBS) (Sigma-Aldrich, Saint Louis, MO, USA) was used as vehicle for both green tea extract containing 45% EGCG was purchased from NutriScience Innovations LLC (Trumbull, CT), and the hydrophilic quercetin conjugates quercetin-3-glucuronide (Q3GlcA) and quercetin-3'-sulphate (Q3'S), were provided by Professor P. W. Needs from the Institute of Food Research (Norfolk, UK).

3.1.3 Flavonoid treatment of LPS stimulated PBMC cultures

Isolated PBMCs were adjusted to a concentration of $1x10^6$ cell/ml with C-RPMI. Cell counts were performed using either a Countess™ automated cell counter (Life Technologies, Grand Island, NY, USA) or by flow cytometry using uniform sized flow-count fluorospheres (Beckman Coulter, Fullerton, CA, USA). Prior to using the automated cell counter 200 µl of a 1:10 dilution of each 5ml cell suspension was prepared in PBS, 10 µl of which was further diluted in an equal volume of supplied trypan blue stain before loading into a disposable chamber slide and read by the cell counter. For absolute count by flow cytometric analysis, 100 µl of each 5 ml cell suspension was combined with 100 µl of flow-count fluorospheres in 1 ml of PBS. Fluorospheres were detected on florescence channel 3 (FL3) of a Beckman Coulter FC-500 flow cytometer with lymphocyte subsets gated using linear forward scatter and side scatter intensity using CXP software (Beckman Coulter, Fullerton, CA, USA). Absolute counts were calculated using the following formula: cells/µl = [(cells counted)/(fluorospheres counted)] x (fluorosphere assayed concentration of 1025).

PBMCs were seeded into flat bottom 24-well culture plates ($1x10^6$ cells/well), flat bottom 96-well culture plates ($1x10^5$cells/well), and 75 cm^2 culture flasks ($1x10^7$ cells/flask) for cytokine, metabolic activity, and western blot analyses, respectively. Quercetin, 45% EGCG enriched green tea extract, quercetin-3-glucuronide (Q3GlcA), and quercetin-3'-sulphate (Q3'S) were diluted 1:6 for each replicate to achieve final concentrations of 1, 2, 10, 25, 40, and 50 μM. PBMCs were incubated at 37°C and 5% CO_2 with 1 hour pretreatment with flavonoids followed by 23 hours of stimulation with 0.1 μg/ml of lipopolysaccharides (LPS) from *Escherichia coli* 055:B5 (Sigma-Aldrich, Saint Louis, MO, USA).

3.1.4 Metabolic activity

Tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)2H-5-tetrazolio]-1,3-benzene disulfonate) (Roche Diagnostics, Indianapolis, IN, USA) is cleaved to formazan by succinate dehydrogenases in metabolically active cells and therefore indicative of cell viability. PBMCs (100 μl, $1x10^6$ cells/ml) used in this assay were cultured in parallel with those used for cytokine analyses, supplementing with the same concentration ranges and combinations of flavonoids (12.5 μl) in a total volume of 125 μl. LPS stimulation (25 μl, 0.1 μg/ml) was performed after 1 hour of the 24 hour incubation period at 37°C, 5% CO_2. WST-1 reagent was added (15 μl/well) 4 hours prior to the end of the incubation period at which point the plate was allowed to incubate at room temperature for 5 minutes before measuring the absorbance of each sample by a microplate (ELISA) reader at 450 nm.

3.1.5 Cytokine quantification by ELISA

The concentration of tumor necrosis factor alpha (TNF-α) in culture medium was determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Standards were prepared by making a 100-fold dilution of the provided recombinant human TNF-α (10,000 pg/ml) in animal serum (diluted stock calibrator) followed by a 4-fold serial dilution for standards ranging from 2500 pg/ml to 0.61 pg/ml. 96-well polystyrene plate pre-coated with monoclonal mouse anti-human TNF-α was equilibrated

with a buffered protein base (assay diluent RD1F) (50 µl/well) before addition of standard, sample, or control (200 µl/well) in duplicate. Following a two-hour room temperature incubation, wells were aspirated and washed by automated cell washer with provided wash solution consisting of buffered surfactant (400 µl/well) 4 times, inverting plates and blotting against paper towels after each wash to ensure complete removal of liquid. A polyclonal antibody against human TNF-α conjugated to horseradish peroxidase was then added (200 µl/well) and plates incubated at room temperature for 1 hour. Previous aspiration/wash steps were repeated before adding substrate solution (200 µl/well), a 1:1 combination of hydrogen peroxide and chromogen, and incubating for an additional 20 minutes in the dark at room temperature. 2 N sulfuric acid (50 µl/well) was added to terminate the reaction and optical density was measured at 450 nm using a microplate (ELISA) reader.

3.1.6 Supernatant cytokine quantification by multi-spot electrochemiluminescence detection

Concentrations of TNF-α, interleukin 6 (IL-6), IL-8, and IL-1β in culture medium were determined using a multi-spot electrochemiluminescence detection assay developed by Meso Scale Discovery (MSD) (Rockville, MD, USA). All reagents, equipment, and consumables were supplied in MSD's human proinflammatory-4 II tissue culture kit and the assay was performed according to the manufacturer's instructions. Standards were prepared by performing a 1:10 dilution of the supplied 1µg/ml stock solution in diluent 2, followed by a 1:4 dilution series from 10000 pg/ml to 2.4 pg/ml. MSD plates pre-coated with capture antibodies for the specific cytokines listed were initially incubated for 30 minutes with diluent 2 (25 µl/well) with vigorous shaking (850 rpm) at room temperature. Standards, samples, and blanks were then added in duplicate (25 µl/well) and incubated at room temperature for 2 hours with vigorous shaking (850 rpm). To remove any unbound antibody plates were washed with PBS containing 0.05% Tween-20 (PBS-T) using an automated plate washer. Detection antibody mix containing antibodies against TNF-α, IL-6, IL-8, and IL-1β, and conjugated with MSD SULFO-TAG was added (25 µl/well) and plates incubated for 2 hours as before. Finally, after an additional three washes in PBS-T, 2X read buffer T was added (150 µl/well) and the plates

analyzed using discovery workbench 3.0 software on a SECTOR Imager 6000 (MSD, Rockville, MD, USA). The minimum detectable concentration of TNF-α was 1.0 pg/mL, IL-6 0.7 pg/mL, IL-8 0.7 pg/mL, and IL-1β 0.4 pg/mL.

3.1.7 Western blot analysis

3.1.7.1 Protein extraction from PBMCs

$1x10^7$ PBMCs were cultured in $75cm^2$ culture flasks, pretreating with a 1:6 dilution of 25 μM Quercetin and 5 μM EGCG alone or in combination in a total of 12.5 ml of C-RPMI for one hour followed by stimulation with LPS (0.1 μg/ml) for 30 minutes. To collect non-adherent lymphocytes, supernatant was centrifuged (400xg, 4°C) for 5 minutes. Lymphocytes and remaining adherent monocytes were each washed in ice cold PBS. Lymphocytes were then pelleted (400xg, 5 minutes, 4°C) before resuspension in 400 uL 1X RIPA buffer (0.15M NaCL; 0.5% sodium deoxycholate; 1% Triton X-100; 0.1% sodium dodecyl sulfate; 50mM Tris-HCl, pH 7.4), containing a broad spectrum halt protease and phosphatase inhibitor cocktail (Pierce, Rockland, IL, USA). RIPA suspensions of lysed lymphocytes were to their respective culture flasks to lyse remaining monocytes, and incubated on ice for 5 minutes. To ensure complete lysis and reduce viscosity, flasks were scrapped, lysate transferred to microcentrifuge tubes, and sonicated for 12 seconds.

3.1.7.2 Protein quantification and preparation

Protein concentration was determined using a BCA protein assay kit according to the manufacturer's instructions (Pierce, Rockland, IL, USA). Concentration of samples was measured relative to an albumin standard at a working range of 20-2,000 μg/ml. Samples, standards, and blanks (25 μl/well) were added in duplicate to a 96-well plate (200 μl/well) in combination with working reagent, a 50:1 preparation of reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartarte in 0.1N sodium hydroxide) and reagent B (4% cupric sulfate). Plates were incubated at 37°C for 30 minutes, then absorbance was measured at 562nm on a microplate (ELISA) reader once the plate had reached room temperature.

Thirty micrograms of total protein was prepared by first ensuring an equivalent loading volume across samples by diluting up to the volume of the most concentrated sample. Diluted protein was then suspended in 3X laemmli buffer consisting of 187.5mM Tris-HCL pH 6.8, 6% w/v SDS, 30% glycerol, 0.03% bromophenol blue, and 150mM dithiothreitol (DTT). Prior to separation by electrophoresis, protein samples were reduced by heating at 95-100°C for 10 minutes, incubated on ice for 5 minutes, and centrifuged at max speed for 5 minutes.

3.1.7.3 Electrophoresis

PBMC protein was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at 200 Volts (V) for 45 minutes. Running buffer used was 25 mM Tris base (EMD Chemicals, Gibbstown, NJ, USA), 192 mM glycine (Sigma-Aldrich, Saint Louis, MO, USA), and 0.1% w/v SDS. Resolving gels consisted of 10% w/v acrylamide:bis 29:1 (EMD Millipore, Billerica, MA, USA), 391 mM Tris-HCl pH 8.8, 3.468mM SDS (Sigma-Aldrich, Saint Louis, MO, USA), and 4.383 mM ammonium persulphate (AMRESCO, Solon, OH, USA). Stacking gels consisted of 6.6% w/v acrylamide:bis 29:1, 0.992M Tris-HCL pH 6.8, 0.1% w/v SDS, 0.1% w/v ammonium persulphate. Both resolving and stacking gels were polymerized with 4 µl TEMED (Fisher Scientific, Fair Lawn, NJ, USA).

3.1.7.4 Protein transfer

Protein was transferred from resolving polyacrylamide gel to a nitrocellulose membrane (BioRad, Hercules, CA, USA) at 100 V for 1 hour at 4°C. Transfer buffer used was 25 mM Tris Base, 192 mM glycine, 0.1% SDS, and 20% v/v methanol (Fisher Scientific, Fair Lawn, NJ, USA). To ensure transfer was complete, the gel was rinsed with deionized water and then incubated in comassie stain for 1 hour at room temperature. To visualize protein on the nitrocellulose membrane, it was immersed in a sufficient amount of Ponceau S stain for 5 minutes with gentle shaking and then rinsed with deionized water until membrane background was white. Following confirmation of protein transfer, the membrane was washed for 5 minutes in 50 ml tricine buffered saline (TBS) and

0.1% Tween 20 (TBS/T). TBS used was diluted from a 10X stock consisting of 200 mM Tris base, 1.369 M sodium chloride (NaCl), with pH adjusted to 7.6 using HCl.

3.1.7.5 Blocking and antibody incubations

For specific detection of phosphorylated proteins, the membrane was blocked for 1 hour at room temperature in TBS/T containing 5% Blotto (nonfat dry milk) (Santa Cruz Biotechnology, Dallas, TX, USA). Then after three 10 minute washes with 50 ml TBS/T, it was probed overnight at 4°C with 1:2000 dilutions of monoclonal rabbit anti-human antibodies directed against phospho-NF-κB p65 (Ser536) and β-actin (13E5) (Cell Signaling Technology, Danvers, MA, USA) in TBS/T containing 5% bovine serum albumin (BSA) (Sigma-Aldrich, Saint Louis, MO, USA). Following the overnight incubation, the membrane was washed again (three 10 minute washes 3 in 50 ml TBS/T) prior to a 1 hour, room temperature incubation with secondary, a 1:6000 dilution of goat anti-rabbit IgG (H+L) conjugated to alkaline phosphatase (AP) in TBS/T containing 5% Blotto.

3.1.7.6 Substrate addition and development

Following a final wash series (three 10 minute washes with 50 ml TBS/T) chemiluminescent detection was achieved using 2 ml Immun-Star chemiluminescent substrate containing 100 µl of enhancer reagent (BioRad, Hercules, CA, USA). This signal was developed using an automated x-ray film developer on Amersham hyperfilm ECL (GE Healthcare Biosciences, Pittsburgh, PA, USA). Film was scanned and intensities of bands measured using ImageJ (National Institutes of Health), normalizing to the β-actin signal.

3.2 *In Vivo* Study

3.2.1 In vivo experimental design

Adult, male C57BL/6J mice (n=116) were purchased from Jackson Laboratory. Protocols were approved by the North Carolina Research Campus Institutional Animal Care and Use Committee (IACUC No 11-007).

Mice were randomly assigned to one of five groups: control, 6.25 mg Quercetin/kg and 1.25 mg EGCG/kg (Q/EGCG low), 12.5 mg Quercetin/kg and 2.5 mg EGCG/kg (Q/EGCG mid), and 25 mg Quercetin/kg and 5 mg EGCG/kg (Q/EGCG high), and 25 mg Quercetin/kg only (Q). Animals were given quercetin/EGCG extract through their food (Bio-Serve Inc.) ad libitum. Within each group, animals were randomly assigned to 3 or 14 days of supplementation, resulting in 10 groups (n=11-12 animals per group). For the last three days of the dosing cycle, mice were run on a treadmill at 14 meters/minute (0% grade) for up to 2.5 hours to induce exercise induced inflammation and oxidative stress. Immediately following exercise on the third day, mice were put under isoflurane anesthesia and euthanized by exsanguination via a needle puncture to the abdominal aorta. Blood was immediately injected into EDTA-coated 1-ml microtainer tubes, and resultant plasma snap frozen in liquid nitrogen and stored at -80°C until analysis.

3.2.2 Plasma quercetin

Total plasma quercetin (quercetin and its primary conjugates) was measured following solid-phase extraction via reverse-phase HPLC with UV detection as previously described[315]. Quercetin conjugates were hydrolyzed by incubating 500 μL plasma aliquots with 10 μL 10% DL-dithiothreitol (DTT) solution. 50 μL 0.58 M acetic acid, 50 μL of a mixture of β-glucuronidase and arylsulfatase, and crude extract from *Helix pomatia* (Roche Diagnostics, Mannheim, Germany) for 2 hours at 37°C. Chromatographic analysis was performed using the Ultimate 3000 HPCL-PDA system (Dionex Corporation, Sunnyvale, CA) with a Gemini C18 column (Phenomenex, Torrance, CA).

3.2.3 Plasma cytokine detection by multi-spot electrochemiluminescence

Concentrations of interferon-γ (INF-γ), IL-1β, IL-10, IL-12p70, IL-6, KC/GRO/CINC (CXCL1), TNF-α in EDTA-treated plasma were determined by an electrochemiluminescence detection assay (MSD, Rockville, MD, USA). All reagents, consumables, and equipment were supplied in MSD's mouse pro-inflammatory 7-plex ultra-sensitive kit and the assay performed according to the manufacturer's instructions. The same procedure was followed as that used for

human tissue culture samples, except that diluent 4 was used in place of diluent 2 and the 7 standards ranged from 10,000 pg/ml to 2.4 pg/ml. The minimum detectable concentration of INF-γ was 0.38 pg/mL, IL-1β 0.75 pg/mL, IL-10 11 pg/mL, IL-12p70 35 pg/mL, IL-6 4.5 pg/mL, CXCL1 3.3 pg/mL, and TNF-α 0.85 pg/mL.

3.3 Statistical Analysis

Cytokine data are expressed as mean ± standard error (SE). Standards generated within MSD assays were screened for a % bias between 80 and 120% before calculating sample concentration using a four-parameter logistic model within discovery benchtop 3.0 (MSD, Rockville, MD, USA). Outliers, defined as observations that were greater or less than 2.5 standard deviations from the respective treatment mean, were eliminated from further statistical analysis. Data were then compared between treatment groups of each study design by using an analysis of covariance (ANCOVA) run in SPSS version 20 (IBM, Armonk, NY, USA), controlling for PBMC metabolic activity and mouse weight loss during run within *in vitro* (EGCG and quercetin, including aglycone and conjugates) and *in* vivo (plasma cytokine and run time) data sets, respectively. *In* vitro metabolic activity data was analyzed using a one-way ANOVA with a post-hoc Bonferroni test conducted relative to baseline stimulated control.

CHAPTER 4: Results

4.1 *In Vitro* Anti-Inflammatory Potential of Quercetin

4.1.1 Effect of quercetin on LPS-stimulated PBMC response

To initially test quercetin's anti-inflammatory potential, an *in vitro* inflammatory response was evoked in human peripheral mononuclear cells (PBMCs) isolated from healthy donors using lipopolysaccharide (LPS), a glycolipid component of the cell wall in gram-negative bacteria. Data from preliminary experiments showed that exposure to 0.1 μg/ml of LPS for 23 hours was sufficient to significantly increase levels of the pro-inflammatory cytokine TNF-α in PBMC supernatants relative to non-stimulated controls (data not shown). When pretreated with quercetin aglycone for 1 hour, PBMCs displayed a dose-dependent reduction in TNF-α release, reaching significance at or above 25 μM ($F=10.700$; $p<0.0001$) (Fig. 1A). Additionally, these doses of quercetin were able to significantly decrease levels of other cytokines indicative of an incurring inflammatory event, such as IL-1β and IL-6, when measured in a multiplex electrochemiluminescent array ($F=11.488$ and 13.675, respectively; $p<0.0001$) (Fig. 1B-C). Levels of IL-8, a major mediator of the inflammatory response that functions as a chemoattractant, were also significantly lower in quercetin treated cells relative to stimulated controls, but only at the highest concentration tested (50μM) (Fig. 1D).

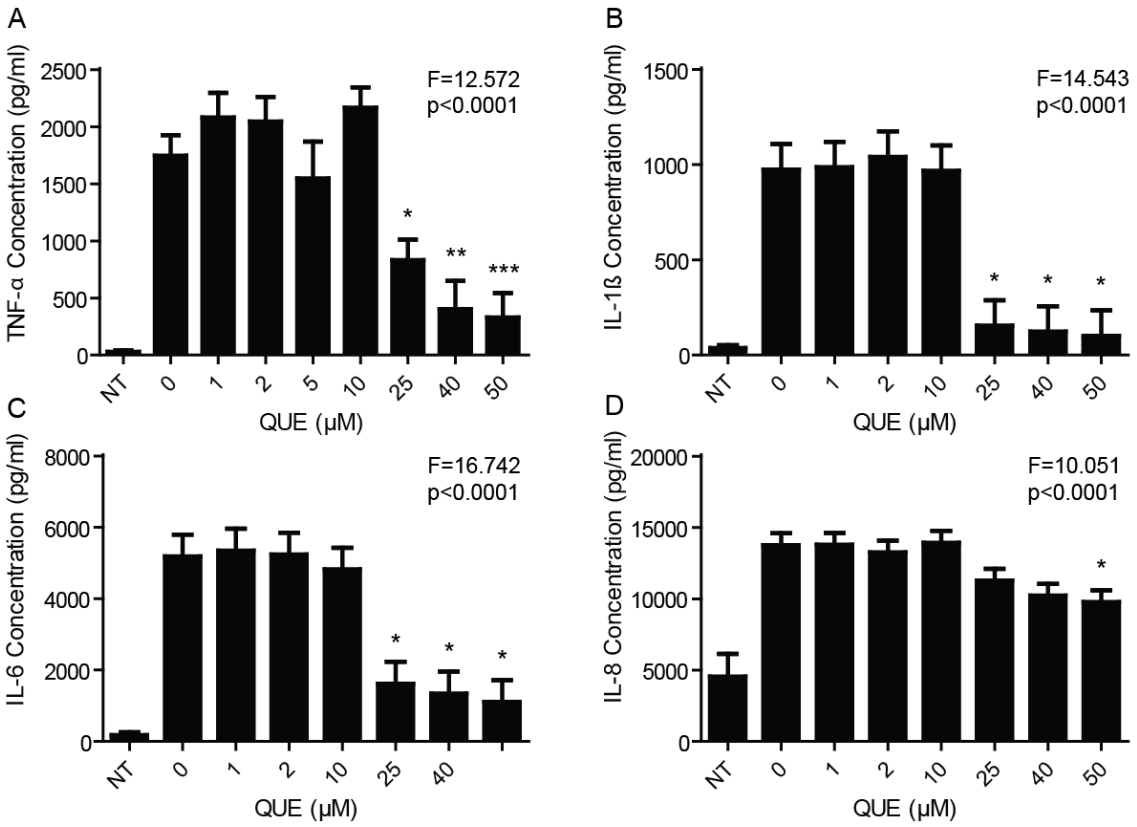

Figure 1. Effect of quercetin (QUE) on LPS induced inflammatory cytokine secretion in human PBMC cutlure. Cells were pretreated with different concentrations of QUE for 1 hour and then stimulated with LPS (0.1 μg/ml) for 23 hours. Levels of TNF-α (**A**), IL-1β (**B**), IL-6 (**C**), and IL-8 (**D**) in culture supernatants were determined by ELISA (**A**) or by a multi-plex electrochemiluminescent detection array (**B**-**D**). Data shown are the average ± SEM of 5 donors measured in duplicate, with exception of TNF-α which includes samples representing an additional 14 donors. An ANCOVA with Bonferroni correction adjusting for PBMC metabolic activity was used to determine whether QUE concentration might independantly affect inflammatory cytokine release in LPS-stimulated PBMCs relative to postive control (represented as 0 μM QUE). (NT=No Treatment) *p<0.05; **p<0.001; ***p<0.0001

4.1.2 Metabolic activity

Metabolic activity was measured in parallel to inflammatory cytokine secretion to determine the effects of QUE treatment on cell viability. Approximately 26.3% (partial eta squared value) of the variability in PBMC metabolic activity could be accounted for by dosage of quercetin supplemented. As shown in Fig. 2, metabolic activity was significantly decreased at 40 and 50 μM QUE when compared to stimulated controls ($F=5.302$; $p<0.0001$). To control for these effects, subsequent inflammatory cytokine secretion data was analyzed by first adjusting for differences in PBMC metabolic activity relative to stimulated, positive controls. Notably, cells treated with 25 μM

QUE maintained a mean relative metabolic activity of 92.58% and after correcting for bias by this covariate, continued to significantly inhibit TNF-α, IL-1β, and IL-6 secretion (Fig. 1,2).

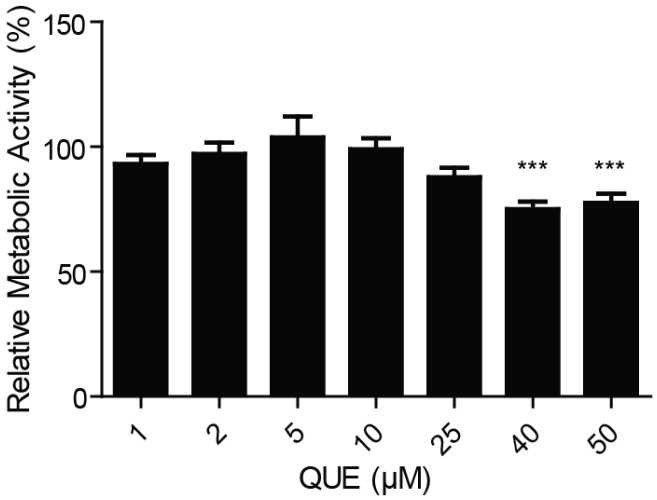

Figure 2. Effect of quercetin (QUE) on PBMC metabolic activity (%) relative to stimulated controls (represented at 100%). Cells were pretreated with different concentrations of quercetin for 1 hour and then exposed to LPS (0.1 μg/ml) for 23 hours, with the addition of WST-1 reagent 4 hours prior to the end of incubaton. Data shown are the average ± SEM of 19 donors measured in triplicate. Statistical significance was determined by one-way ANOVA with a post-hoc Bonferroni test conducted relative to baseline stimulated control. ($F=6.361$, $p<0.0001$). ***$p<0.0001$

4.1.3 Effect of quercetin conjugates on LPS-stimulated PBMC response

It is generally accepted that ingestion of quercetin in either its glycoside or aglycone form must undergo significant modification prior to its bioavailability in the systemic circulation. Upon absorption in the small intestine and subsequent processing by the liver, the aglycone can be modified by conjugation reactions, such as sulfation and/or glucuronidation, and by methylation of hydroxyl groups present on its catechol-ring by the enzyme catechol-*O*-methyltransferase. Therefore, this study tested, two of the three most abundant human plasma quercetin conjugates, quercetin-3-glucuronide (Q3GlcA) and quercetin-3'sulfate (Q3'S), in the *in vitro* LPS-stimulated PBMC model previously established. Neither Q3GlcA nor Q3'S at concentrations ranging from 1-40 μM significantly inhibited LPS-induced inflammatory cytokine secretion when compared to untreated controls (Fig. 3-4). Parallel measurements of metabolic activity indicated that greater than 95% of the cells remained viable at the highest concentration of either conjugate compared to baseline,

stimulated controls (Fig. 5). These data were anticipated since conjugation decreases a flavonoid's lipophilicity and would not favor their passive diffusion through PBMC cellular membranes. In the absence of some unknown, specific transport system or interaction with a yet unidentified surface receptor, the hydrophilic nature of Q3GlcA and Q3'S would likely prevent any effects these metabolites may have on PBMC homeostasis. Supplies of Q3GLcA and Q3'S were limited thus concentrations as high as 50µM could not be tested.

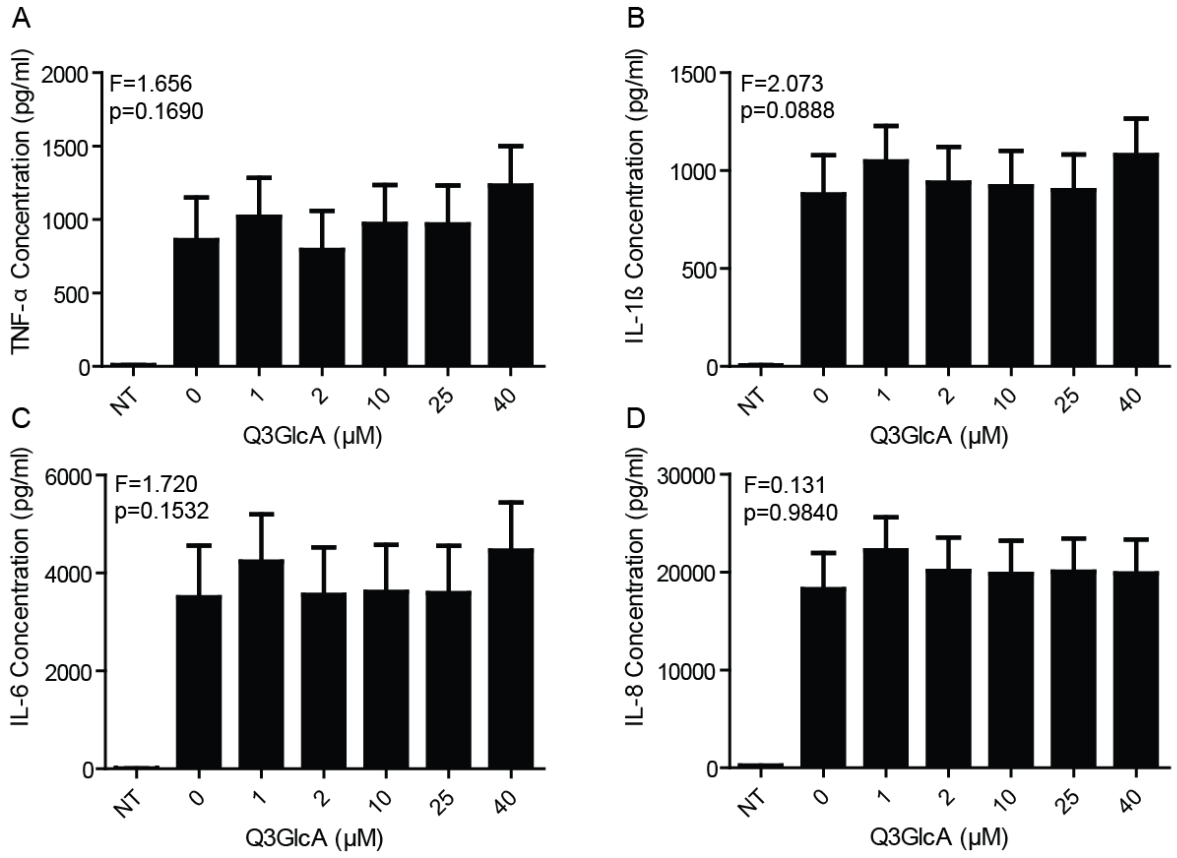

Figure 3. Effect of quercetin-3-glucuronide (Q3GlcA) on LPS-induced inflammatory cytokine secretion in human PBMC culture. Cells were pretreated with or without Q3GlcA ranging in concentration from 1-40 µM for 1 hour followed by 23 hours of exposure to LPS (0.1 µg/ml). Levels of TNF-α (**A**), IL-1β (**B**), IL-6 (**C**), and IL-8 (**D**) were determined by a multi-plex electrochemiluminescent dection array. Data shown are the average ± SEM of 6 donors measured in duplicate. An ANCOVA with Bonferroni correction was performed t to adjust for effects of Q3GlcA on PBMC metabolic activity. (NT=No Treatment)

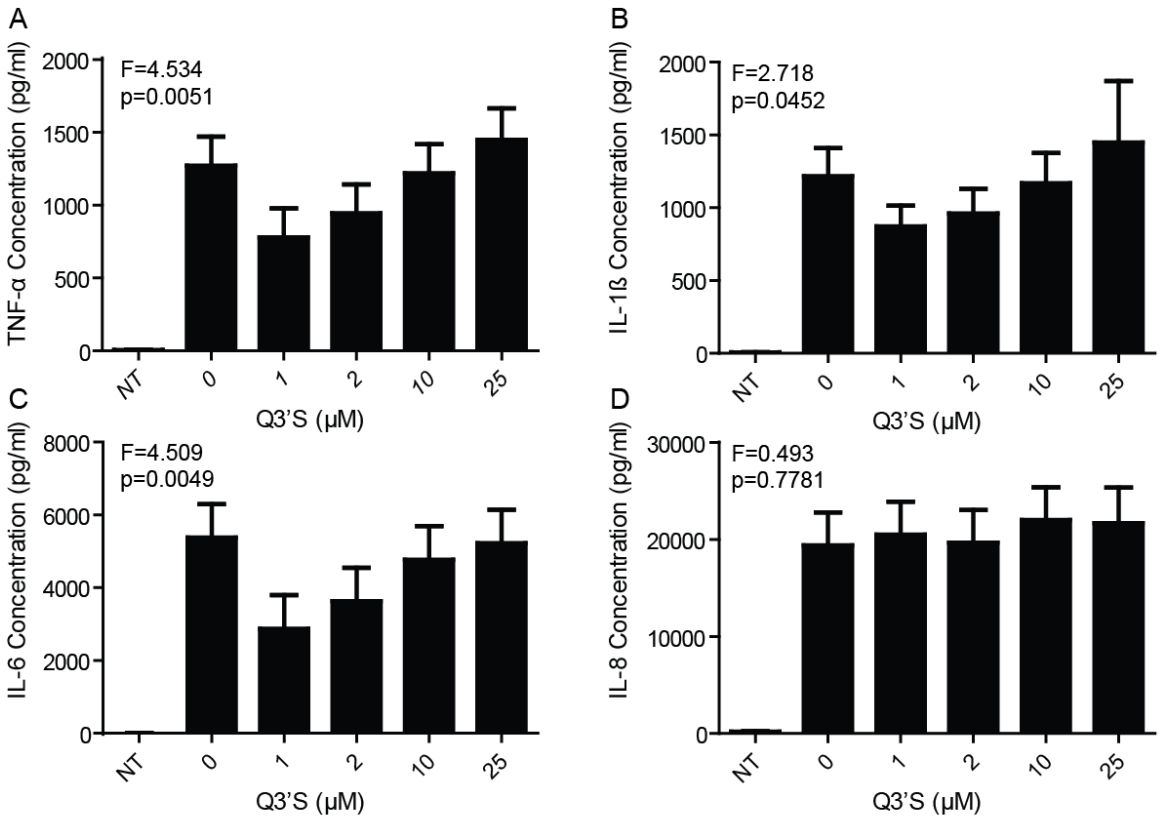

Figure 4. Effect of quercetin-3'-sulfate (Q3'S) on LPS-induced inflammatory cytokine secretion in human PBMC culture. Cells were incubated with or without Q3'S ranging in concentration from 1-25 μM and then exposed to LPS (0.1 μg/ml) for an additional 23 hours. Levels of TNF-α (**A**), IL-1β (**B**), IL-6 (**C**), and IL-8 (**D**) in culture supernatants were determined by a multi-plex electrochemiluminescent dection array. Data shown are the average ± SEM of 6 donors measured in duplicate. An ANCOVA with Bonferroni correction was performed to adjust for effects of Q3'S on PBMC metabolic activity. (NT=No Treatment)

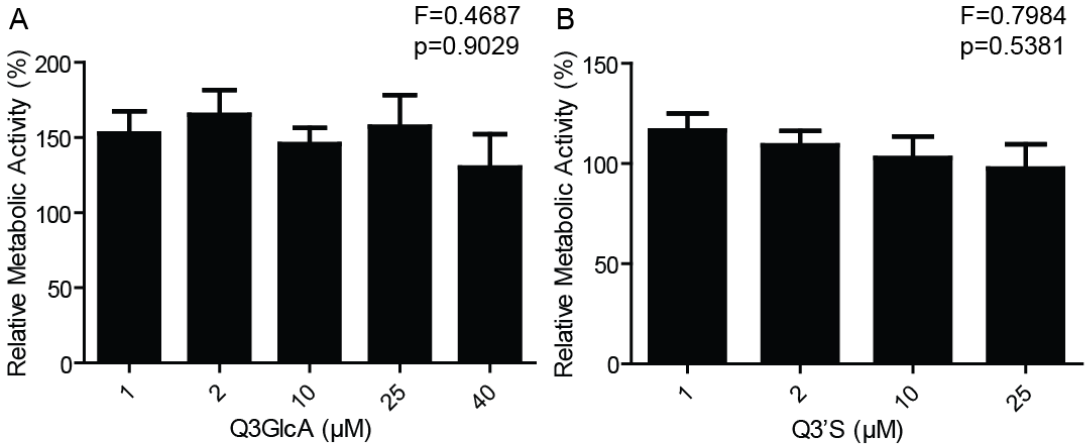

Figure 5. Effect of quercetin-3-glucronide (Q3GlcA) (**A**) and quercetin-3'-sulfate (Q3'S) (**B**) on PBMC metabolic activity (%) relative to stimulated controls (represented at 100%). Cells were pretreated with Q3GlcA or Q3'S for one hour prior to 23 hours of stimulation with LPS (0.1 μg/ml), with the addition of WST-1 reagent when only 4 hours remained. Data shown are the average ± SEM of 6 donors measured in triplicate. Statistical significance was determined by one-way ANOVA using a post-hoc Bonferroni test to compare treatment groups against baseline stimulated controls.

4.1.4 Quercetin & EGCG in combination: Assessing putative synergistic effects

Previously it has been shown that endurance athletes receiving 1000-mg quercetin per day for up to 3 weeks failed to counter exercise-induced inflammation incurred during the heavy exertion of competition[8]. However, only two weeks of quercetin supplementation was sufficient to significantly decrease C-reactive protein (CRP) and plasma levels of IL-6 and the anti-inflammatory mediator IL-10 following a three-day period of intense exercise when combined with EGCG, isoquercetin, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)[268]. To further explore how the addition of these compounds might improve quercetin's bioactive effects, EGCG was combined with quercetin at different ratios to supplement *ex vivo* human PBMCs. Ratios tested included QUE (μM): EGCG (μM) at 5:5, 10:5, 10:10, 25:10, and 25:25. As shown in Fig. 6, none of the quercetin to EGCG ratios tested significantly inhibited TNF-α secretion from LPS-stimulated PBMCs. On average 25 μM of quercetin combined with 10 μM of EGCG was sufficient to cause a 3.89 fold decrease in TNF-α, yet this failed to be significant after adjusting for metabolic activity.

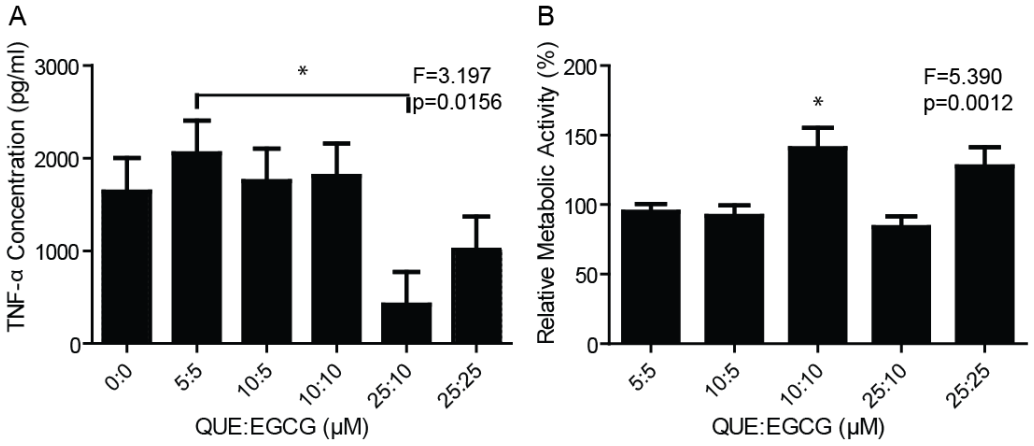

Figure 6. The effect of supplementing with quercetin (QUE) and EGCG in combination on TNF-α secretion (**A**) and metabolic activity (**B**) in LPS stimulated human PBMCs. Experiments were conducted in parallel, using cells pretreated with or without varying concentrations of quercetin and EGCG for 1 hour followed by exposure to LPS (0.1 μg/ml) for an additional 23 hours. Concentrations of TNF-α (**A**) measured by ELISA and % metabolic activity (**B**) were the average ± SEM of 6 donors measured in duplicate and triplicate, respectively, with metabolic activity calculated relative to values of stimulated controls set at 100%. To determine if flavonoid supplementation was an independent indicator of TNF-α secretion, an ANCOVA was performed with Bonferroni correction, with adjustment for differences in PBMC metabolic activity. Stastical significance of relative metabolic activity was determined using a one-way ANOVA with post-hoc Bonferroni test conducted relative to baseline stimulated control. *p<0.05

To assess the effects of EGCG alone on PBMC inflammatory response or viability, EGCG was added without quercetin at different concentrations. EGCG dose dependently increased TNF-α levels in PBMC supernatant after 23 hours of incubation with LPS, which reached significance ($p<0.001$) at the highest concentration tested (50 μM) (Fig. 7A). Accordingly, metabolic activity relative to stimulated controls was significantly reduced when cells were pretreated with either 25 or 50 μM EGCG (Fig. 7B). These data suggest that EGCG supplemented in this culture system has a negative impact on cell viability and can potentially amplify an inflammatory response at doses at or greater than 50 μM.

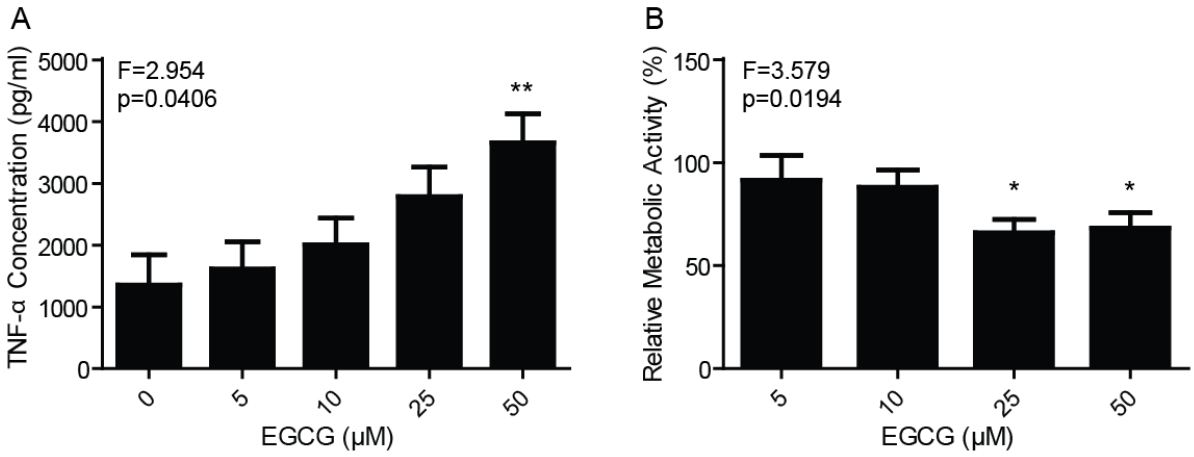

Figure 7. Effect of EGCG on TNF-α secretion (**A**) and metabolic activity (**B**) in LPS-stimulated human PBMCs. Experiments were conducted in parallel using cells pretreated with different concentrations of EGCG for 1 hour followed by LPS stimulation (0.1 μg/ml) for 23 hours. Levels of TNF-α (**A**) quantified by ELISA and % metabolic activity (**B**) were the average ± SEM of 6 donors measured in duplicate and triplicate, respectively, with metabolic activity calculated relative to values of stimulated controls set at 100%. An ANCOVA with Bonferroni correction, adjusting for differences in PBMC metabolic activity, was performed to determine whether or not EGCG supplementation could independentaly predict TNF-α secretion. Stasticial significance of relative metabolic activity was determined by one-way ANOVA with post-hoc Bonferroni conducted relative to baseline stimulated control. *p<0.05 **p<0.001

4.2 *In Vivo* Anti-Inflammatory Potential of Quercetin

4.2.1 Selection of a quercetin to EGCG ratio based on in vitro findings

Despite the fact that *in vitro* results for this study provided no direct evidence to substantiate claims that EGCG was necessary to improve quercetin's effectiveness as an anti-inflammatory agent in humans, this did not rule out a role for EGCG in quercetin bioavailability. Herein, quercetin has

been shown to decrease expression of inflammatory markers at the high concentration of 25 μM, without significantly affecting cell viability. Observations of similar anti-inflammatory action *in vivo* may only require improvements on its dietary absorption and accumulation in tissue rather than synergistically amplifying its bioactive effects within cells.

To determine if coingestion of EGCG was required to obtain anti-inflammatory effects from quercetin supplementation, its impact would need to be measured using an *in vivo* experimental design. Due to EGCG's detrimental effect on cell viability with increasing concentration in culture, its dose relative to quercetin was kept minimal. With that in mind, a 5:1 ratio of quercetin to EGCG was selected to formulate diets to dose C57BL/6J mice. *In vitro* testing of this ratio was performed prior to the animal study to ensure that EGCG did not affect quercetin's bioactive effects on LPS-stimulated PBMC response. Additionally, 25 μM of quercetin was used in this final cell culture experiment since this concentration had previously caused a significant decrease in TNF-α secretion when correcting for metabolic activity. As seen in Fig. 8A-C, QUE and EGCG at a ratio of 5:1 significantly decreased secretion of both IL-1β and IL-6 from PBMCs after adjusting for metabolic activity. In this experiment QUE failed to significantly decrease TNF-α secretion, which was likely due to the sample size being limited to 5 donors compared to prior experiments, which were representative of 14 donors. On average TNF-α secretion from PBMCs supplemented with QUE and EGCG combined approximated that of QUE alone. Taken together, these results indicated that additional supplementation of EGCG when concentration was kept five times lower then that of QUE, had no adverse effect QUE's bioactive effects *in vitro*.

LPS is known to stimulate the canonical activation of transcription factor NF-kB via toll-like receptor 4 (TLR-4) to cause upregulated expression of TNF-α, IL-6, and IL-1β in macrophages. Furthermore, it has been demonstrated that LPS stimulation can induce phosphorylation of serine 536 on the trans-activation domain of NF-κB subunit p65 in an IKK-dependent manner to increase transcriptional activity. To determine if QUE supplementation affected NF-κB transcriptional activity in this manner, PBMC protein lysates were probed with a monoclonal antibody specific to

p65 only when phosphorylated at serine 536. As seen in Fig. 8D, QUE alone or combined with EGCG was able to decrease p65 phosphorylation in LPS-stimulated PBMCs to baseline levels observed in un-stimulated controls.

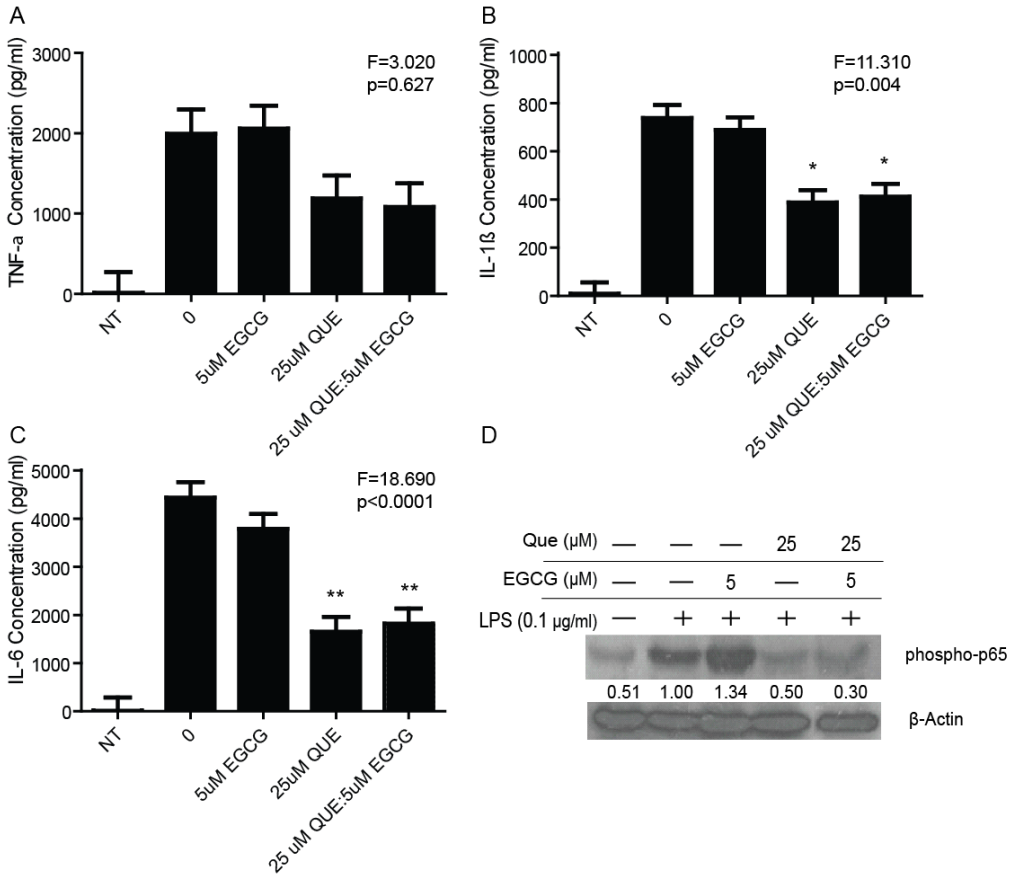

Figure 8. Effect of quercetin (QUE) and EGCG at a ratio of 5:1 on the secretion of inflammatory cytokines TNF-α (**A**), IL-1ß (**B**), IL-6 (**C**), and phosphorylation of p65 (**D**), a subunit of transcription factor NF-κB. Cells were pretreated with either 25 µM of QUE or 5 µM of EGCG alone or in combination for 1 hour followed by stimulation with LPS (0.1 µg/ml) for 23 or 0.5 hours for (**A**-**C**) and (**D**), respectively. Cytokine concentration as quantified by a multi-plex electrochemiluminescent dection array are the average ± SEM. of 5 donors measured in duplicate. An ANCOVA with Bonferroni correction was performed to adjust for the effects of QUE or EGCG on PBMC metabolic activity. The phospho-p65 (**D**) immunoprecipitate was resolved on 10% SDS-PAGE, transferred onto nitrocellulose membrane and probed with anti-phospho NF-kB p65 (Ser536), 93H1 and anti-b-Actin, 13E5. Data shown are results relative to a positive control (represented at 1.00) from a single experiment and are normalized to levels of β-actin. (NT=No Treatment) *p<0.05, **p<0.001

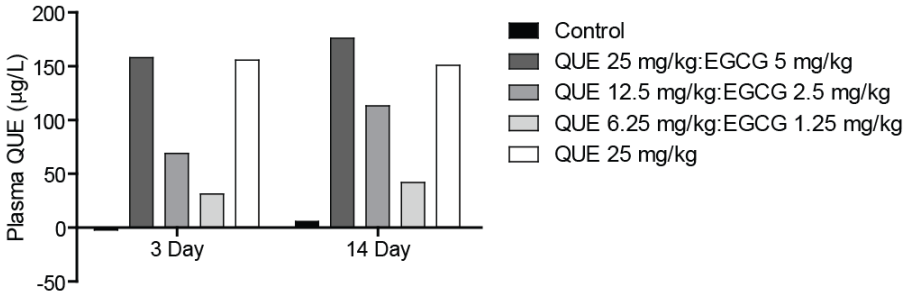

Figure 9. Plasma quercetin (QUE) after 3 and 14 day supplementation in C57BL/6J mice following 3 consecutive days of treadmill running. Data shown were measured from pooled plasma samples from each treatment group from mice undergoing either 3 or 14 day dosing cycles.

4.2.2 QUE and EGCG supplementation in mice

Diets were formulated for C57BL/6J mice with quercetin concentrations at 25, 12.5, and 6.25 mg/kg combined with 5, 2.5, and 1.25 kg/mg EGCG at a 5:1 ratio, with an additional treatment group receiving only quercetin at the high 25 mg/kg concentration. Plasma quercetin levels were similar even at the highest 25 mg/kg concentration regardless of supplementation length in the absence of EGCG at 155.71 and 151.00 ug/L for 3 and 14-day dosing, respectively. However, when plasma quercetin levels for treatment groups receiving EGCG were compared, 14-day supplementation tended to have higher plasma quercetin levels then 3-day (Fig. 9).

4.2.3 Post-exercise inflammatory response following QUE and EGCG supplementation

Intense and prolonged exercise has been shown to increase levels of both pro- and anti-inflammatory cytokines in the plasma of both animals and humans alike. To determine if QUE alone or combined with EGCG at a ratio of 5:1 had any effect on this post-exercise cytokine response, male C57BL/6J completing either 3- or 14-day dosing cycles participated in treadmill running for 2.5 hours or until exhaustion for 3 consecutive days. Fig. 10 shows plasma cytokine levels immediately following the final exercise bout after adjusting for weight loss during the run. Of all the cytokines measured, only IL-10 was significantly decreased after exhaustive exercise in mice supplemented for 14 days. However, these results remain inconclusive since IL-10 measured in the 14-day flavonoid supplementation group failed to significantly differ from either 3-day control or supplemented mice.

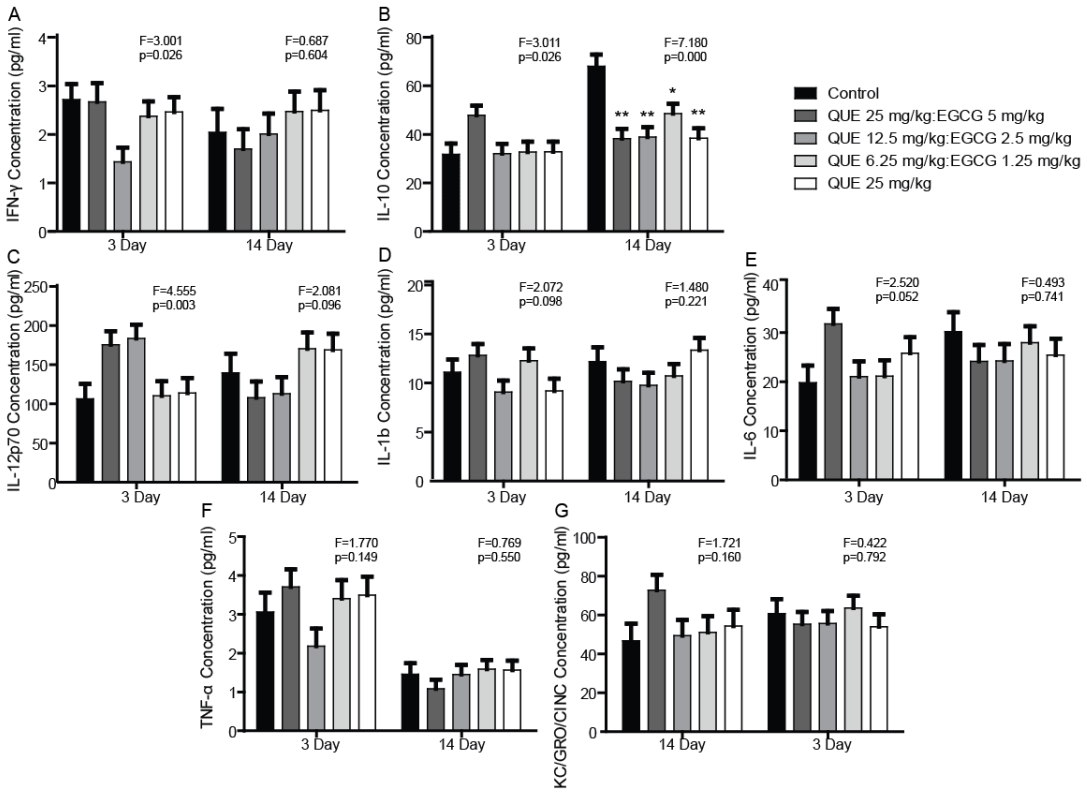

Figure 10. Postexercise plasma cytokine levels after 3 and 14 day supplementation of quercetin (QUE) alone or in combination with EGCG at a 5:1 ratio. Following the designated dosing cycle, C57BL/6J mice (n=116) completed 2.5 hours of treadmill running for 3 consecutive days (n=11-12 mice per treatment group). Immediately following exercise on the third day, mice were put under isofluorine anesthesia and exsanguinated by cardiac puncture. Plasma cytokines from EDTA-treated blood were were measured by 7-plex elecrochemiluminescent dection array. To determine if cytokine secretion was an independent indicator of flavonoid supplementation relative to control diet, an ANCOVA with Bonferroni correction was performed adjusting for differences in weight lost during the final 2.5 hour running bout. *p<0.05, **p<0.001

4.4.4 QUE's effect on exercise performance when combined with EGCG

The extent to which mice were allowed to run on a given day in this study was capped at 2.5 hours. However, since mice were naïve to this level of exercise, many fatigued before completing the maximal running time, which occurred more often on day 1 compared to day 3 of exercise. For example, 14-day supplemented mice along with control ran on average for 135.2, 143, and 149.5 minutes for exercise day 1, 2, and 3, respectively. To see if diet had any effect on running performance over the course of the three exercise days, the difference in average run time of supplement groups was measured relative to control (Fig. 11). After adjusting for weight loss, neither 3-day (F=1.312, p=0.2779) nor 14-day (F=2.331, p=0.0677) supplemented groups showed significantly improved run time.

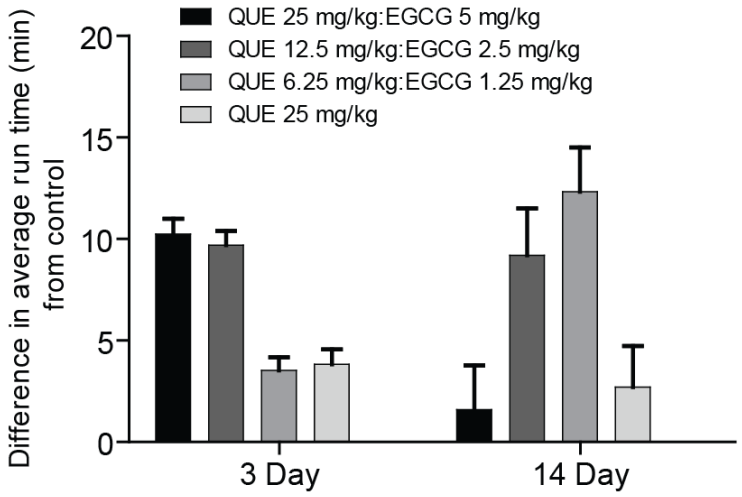

Figure 11. Difference in average run time (min) following 3 or 14 day supplemenation with quercetin (QUE) alone or in combination with EGCG at a 5:1 ratio relative to control diet. C57BL/6J mice were randomly assigned to treatment groups and following designated dosing cycle, underwent 3 consecutive days of treadmill running for 2.5 hours or until exhaustion. An ANCOVA with Bonferroni correction adjusting for weight lost during exercise bout was performed to determine if supplemental dosing could indenpendently predict prolonged, endurance exercise performance.
(3 day: F=1.312, p=0.2779; 14 day: F=2.331, p=0.0677)

CHAPTER 5: Discussion

Prolonged and intensive exercise, such as marathon and ultramarathon race events, intensify the inflammatory response within working muscle, resulting in tissue damage, systemic inflammation, and marked increases in oxidative stress[4,316,317]. To this effect, responding *de novo* translation of pro-inflammatory cytokine genes, such as IL-6, IL-8, IL-1β, and TNF-α, and a key enzyme in prostaglandin production cyclooxygenase-2 (COX-2) have been measured in abundance in post-exercise muscle biopsies in humans, with 20 to 30-fold increases reported in IL-6 and IL-8 mRNA[4,7,8]. Furthermore, it is speculated that IL-1β can induce NF-κB regulated expression of IL-6 in an autocrine or paracrine fashion within muscle that is distinct from its expression in response to contraction via JNK[318]. This degree of cytokine expression and secretion, along with profound increases in hormones, such as cortisol, and local and systemic oxidative stress, is said to be indicative of the degree of physiological stress that could predispose an athlete to increased infection risk. Athletes have in the past been known to take medication for this very reason. However, over the counter nonsteroidal anti-inflammatory medications (NSAIDS), such as ibuprofen, have been shown to increase gastrointestinal permeability and endotoxemia following prolonged endurance events[4,13].

Blunting exorbitant inflammation in athletes may be an effective means to counter exercise-induced alterations in immune status, improving overall recovery and subsequent performance. Flavonoids offer an attractive alternative to these medications since they have not been linked to any adverse side effects in athletes and are strong antioxidants with putative anti-inflammatory action[319]. Numerous *in vitro* studies have been conducted on the anti-inflammatory potential of quercetin, a flavonoid with metal-chelating ability and high antioxidant capacity. The present study has confirmed previous findings of quercetin's anti-inflammatory capacity *in vitro*[250,253,254]. Concentrations of the aglycone supplement tested at or greater than 25 μM significantly decreased secretory levels of TNF-α, IL-6, and IL-1β in *ex vivo* derived human PBMCs following LPS stimulation.

Despite quercetin's well-established immune modulating activity *in vitro*, to date similar findings in an *in vivo* setting have yet to be reported. In two separate studies, cyclists receiving 1,000 mg of quercetin per day for three weeks failed to counter inflammatory or oxidative perturbations post-exercise compared to placebo[8,286]. A plausible explanation for this discrepancy may lie in the pharmacokinetics of the compound. Following intestinal uptake and hepatic processing, circulating forms of the polyphenol exist as conjugated derivatives of the ingested aglycone. Numerous studies have reported changes in biological activity upon metabolic transformation of flavonoids[188,227,320–322]. Indeed, the presence of a 3' hydroxyl group within the B-ring and a 2,3-double bond within the C-ring of quercetin's phenylbenzopyrone structure has been reported to have a profound effect on its ability to counter leukotriene B_4 (LTB_4) formation in *ex vivo* derived human, peripheral monocytes and neutrophils [323]. In a similar manner, the present study has established that metabolic derivatives of quercetin, quercetin-3'-sulphate and quercetin-3'-*O*-glucuronide, exhibit a diminished bioactive effect on LPS stimulated pro-inflammatory cytokine production in *ex vivo* derived human PBMCs. The precise mechanism by which conjugation in this manner diminishes the anti-inflammatory activity of these metabolites cannot be determined based on these experiments. However, the addition of polar moieties such as glucuronide and sulfate has been reported to significantly diminish cellular uptake of quercetin in neutrophils[323]. Furthermore, it has been recently noted that the majority of ingested quercetin and other polyphenolics reach the colon where resident bacteria degrade them to smaller phenolics. A human metabolomics profile on serum collected immediately following three days of running 2.5 hours/day, 70% VO_{2max} revealed a significant increase in these phenolics compared to 14 hours post-exercise. It is conceivable that the anti-inflammatory effect exhibited by Q-EGCG was elicited by these smaller phenolics and dependent on their accumulation in tissue[324].

Another explanation for quercetin's poor effect *in vivo* may be linked to the bioavailability of the compound. Intestinal absorption of dietary quercetin aglycone is said to be at most 24%, with plasma levels ranging from 0.9 to 7 μM, well below concentrations shown to effectively reduce pro-

inflammatory cytokine production *in vitro*[8,234,266,268,269,273–275,286]. Consistent with these previous findings, plasma levels in the present study reached only as high as 150 mg/L or 0.5 μM in C57BL/6J mice supplemented on 25 mg/kg quercetin for either 3 or 14 days. It has been suggested that bioavailability of flavonoids may be enhanced when combined in supplementation with other nutritional bioactives[161,195,325]. Indeed, a previous study found enhanced immunomodulatory effect post-exercise in cyclists that had been taking Q-EGCG, a supplement combining quercetin with vitamin C, isoquercetin, epigallocatechin gallate (EGCG), and n-3 fatty acids, for two weeks daily[324].

One caveat of the *in vivo* model described herein is that resting peripheral blood cytokine measures were not taken. To do this would have required more mice assigned to each diet serving as a non-exercise control. Without this control, the lack of significant difference in cytokine measures between supplemented and placebo diets could be explained by the simple fact that said cytokine may have not been elevated above homeostatic levels following exercise. For instance, no human studies have shown an increase in plasma TNF-α immediately following exhaustive exercise, instead only reporting increases several hours post-exercise[326–330]. Of the seven cytokines measured, only IL-10 was lower in quercetin supplemented versus control mice immediately following exercise after adjusting for weight loss. IL-10 is a cytokine associated with immunosuppression since it attenuates the synthesis of IL-1α, IL-1β, TNF-α, IFN-γ, IL-6, IL-8, CCL3, CCL4, G-CSF, and GM-CSF and augments IL-1ra production[331,332]. Numerous studies have reported elevated plasma IL-10 levels immediately following exhaustive exercise[18,333–336]. Its downregulation following 14-day quercetin supplementation in this study is in agreement with that found in the Q-EGCG chew tested in humans after exhaustive cycling. However, 14-day supplementation with Q-EGCG also decreased post-exercise levels of plasma IL-6, a finding that was not reproduced in this study[268].

Putative anti-inflammatory effects have also been described in both animal and *in vitro* models testing EGCG, a catechin and predominate polyphenol in green tea extract [216,337–339]. To test whether EGCG could be contributing to the enhanced bioactive effects of Q-EGCG, mice in the

present study were given quercetin and EGCG at a 5:1 ratio. This ratio was chosen based on the *in vitro* finding that a high to low proportion of quercetin to EGCG was most effective at blunting LPS-stimulated TNF-α production in PBMCs. Furthermore, EGCG was found to dose-dependently increase TNF-α production, with a detrimental effect on cell viability at 25 and 50 μM concentrations. These results were in agreement with findings by Crouvezier et al. (2000), who also reported EGCG's negligible effect on LPS-stimulated TNF-α production in human PBMCs at concentrations of up to 20 μM[339]. Unfortunately, results from the present animal study shed doubt that the combination of flavonoids in Q-EGCG contributed to its effectiveness in human athletes. In fact, none of the seven cytokines measured post-exercise differed between quercetin only treated mice and those receiving a 5:1 combination of quercetin and EGCG. Since the *in vitro* experiment determining the optimal ratio of quercetin to EGCG failed to have a quercetin only control, these experiments were repeated using 25 μM quercetin and 5 μM EGCG, both alone and in combination. Results from this experiment confirm that 5 μM EGCG failed to enhance the anti-inflammatory effect of quercetin in the scope of this model.

Another caveat of this study was that the assessment of anti-inflammatory effectiveness in exercised mice was limited to plasma cytokine measures. In a more recent study of obese women supplemented on a mixed flavonoid-nutrient-fish oil supplement (Q-Mix; 1000 mg quercetin, 400 mg isoquercetin, 120 mg EGCG from green tea extract, 400 mg *n3*-PUFAs from fish oil, 1000 mg vitamin C, 40 mg niacinamide, and 800 μg folic acid) for 10-weeks, similar biomarkers of inflammation (CRP and cytokine panel) were not altered, however further microarray gene expression analysis revealed overrepresentation of 27 inflammatory response-related bio-functions in Q-mix versus placebo. Since assessment of anti-inflammatory effectiveness in the current study was limited to whole blood cytokine levels, it is conceivable that quercetin may have influenced expression of other immunomodulatory genes. In addition, peripheral blood protein and gene expression fails to capture the dynamic paracrine and autocrine signaling occurring between infiltrating leukocytes and myofibers in inflamed skeletal muscle tissue.

In conclusion, aims accomplished *in vitro* in this thesis study included comparing quercetin's anti-inflammatory potential to that of biologically relevant quercetin conjugates, and both understand and optimize the ratio at which quercetin and EGCG combined exerts the most potent anti-inflammatory potential. Analysis conducted on the thesis *in vitro* data indicated that conjugation with either glucuronide or sulfate eliminates quercetin aglycone's anti-inflammatory effect *in vitro*. However, EGCG exerts a pro-inflammatory effect at high concentrations within this *in vitro* model and, when combined with quercetin aglycone, fails to potentiate quercetin's anti-inflammatory effect. Nevertheless, a 5:1 ratio of quercetin to EGCG was chosen for subsequent testing in an exercising mouse model, reasoning that a low dose of EGCG relative to quercetin aglycone would not counteract any putative, anti-inflammatory effect of quercetin *in vivo*. The *in vivo* thesis data herein failed to demonstrate a requirement for EGCG in substantiating quercetin's effect on post-exercise inflammation in mice. Furthermore, EGCG at the relative concentrations tested failed to impact the bioavailability of quercetin in mice.

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Vita

Krista M Kennerly was born in Des Moines, Iowa. She is the daughter of Helen Patrice Wallenborn and Robert Michael Kennerly. Krista attended elementary, middle, and high school in Asheville, NC and then moved to Boone, NC to earn a Bachelor of Science in Biology with an additional minor in Chemistry from Appalachian State University in 2009. During her senior year as an undergraduate she began independent study in Dr. Dru Henson's laboratory. Following graduation, she decided to continue her mentorship under Dr. Henson in pursuit of her Master of Science in Biology with a concentration in Cell and Molecular Biology. As a master's student she was a Kannapolis Scholar and was supported in part by a fellowship provided by Agriculture and Food Research Initiative Grant no. 2010-65200-20354 from the USDA National Institute of Food and Agriculture. This fellowship enabled her to expand her research pursuits as a master's student and allowed her to conduct research at the North Carolina Research Campus during the summers of 2010 and 2011. In the fall of 2011, she also received research grants from the Graduate Student Association Senate and Office of Student Research. After completing her master's research, she moved to Saint Louis, Missouri where she currently works as a senior research technician in Dr. Deepta Bhattacharya's lab at Washington University School of Medicine.